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In vitro micropropagation of three orchid accessions (*Orchidaceae*) in the BIORENA Germplasm Bank

Propagação *in vitro* de três acessos de orquídeas (*Orchidaceae*) no Banco de Germoplasma BIORENA

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Highlights

Native orchids can be propagated efficiently *in vitro.*
The germplasm bank conserves endangered orchid species.
Media optimization improves rooting and survival rates.

Abstract

Native orchids are increasingly fragile in terms of survival within ecosystems that are being progressively altered by deforestation, land-use changes, illegal trade, and many others factors. It is estimated that the number of orchids in Bolivia ranges between 2,000 and 3,000, considering both identified and unidentified species. Their unique flowering makes it essential to study them for conservation purposes. Within this framework, this research was conducted at the BIORENA Biotechnology Laboratory to propagation three accessions collected with capsule of native orchids (*Orchidaceae*) by establishing protocols for *in vitro* seed micropropagation during the establishment and multiplication phases lacking the rooting and actual conservation phases. Seed capsules were collected in October 2023 in the forest at community of San Juan de Saguayo, near Amboró Park in Bolivia. For the establishment phase, the seeds were disinfected with sodium hypochlorite (NaOCl) at 5%, 7% and 10%. A two-factor design with a completely randomized arrangement was used. Three orchid accessions (BAO-001, BAO-002, BAO-003) and three hypochlorite concentrations (5%, 7% and 10%) were used in establishment phase. During the multiplication stage, three MS medium formulations were evaluated: (1) MS supplemented with activated charcoal (AC) and indole-3-acetic acid (IAA), (2) MS supplemented with pyridoxine (P), and (3) MS supplemented with gibberellic acid (GA_3). The treatment with 100% germination was achieved with 10% sodium hypochlorite. The survival rate of the accessions was 77.79%, and in the multiplication stage, the survival rate reached 100% at 45 days. In conclusion the MS + GA_3 medium in accession BAO-

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003 showed to be of larger size, with a greater number of leaves and a greater number of roots.
Key words: Micropropagation. Orchids. *In vitro*. Gibberellic acid. Sodium hypochlorite.

Resumo

As orquídeas nativas são cada vez mais vulneráveis em termos de sobrevivência nos ecossistemas, que vêm sendo progressivamente alterados pelo desmatamento, mudanças no uso da terra, comércio ilegal e outros fatores. É estimado que o número de espécies de orquídeas na Bolívia variar entre 2.000 e 3.000, considerando as espécies identificadas e não identificadas. Sua floração única torna essencial o estudo dessas plantas para fins de conservação. Nesse contexto, esta pesquisa foi conduzida no Laboratório de Biotecnologia do BIORENA para propagar três acessos colectados com capsulas de orquídeas nativas (*Orchidaceae*), estabelecendo protocolos para micropropagação *in vitro* de sementes durante as fases de estabelecimento e multiplicação, carente das fases de enraizamento e conservação propriamente dita. As cápsulas de sementes foram coletadas em outubro de 2023 na floresta da comunidade de San Juan de Saguayo, próxima ao Parque Amboró, na Bolívia. Para a fase de estabelecimento, as sementes foram desinfetadas com hipoclorito de sódio (NaOCl) a 5%, 7% e 10%. Foi utilizado um delineamento de dois fatores com arranjo completamente aleatorizado. Foram avaliados três acessos de orquídeas (BAO-001, BAO-002, BAO-003) e três concentrações de hipoclorito (5%, 7% e 10%) na fase de introdução. No momento da micropropagação, foram utilizados meios de cultura Murashige e Skoog (MS) suplementados com Carvão Ativado (CA) + Ácido Indol-3-Acético (AIA), MS + Piridoxina (P) e MS + Ácido Giberélico (GA_3). O tratamento com 100% de germinação foi obtido com hipoclorito de sódio a 10%. A taxa de sobrevivência dos acessos foi de 77,79%, e na fase de multiplicação a taxa de sobrevivência atingiu 100% a os 45 dias. Em conclusao, o meio MS + GA_3 na acesso BAO-003 apresentou o tamanho maior, com maior número de folhas e raízes.
Palavras-chave: Micropropagação. Orquídeas. In vitro. Ácido Giberélico. Hipoclorito de sódio.

Introduction

The orchidaceae family is a large, diverse, and extensive group of flowering plants worldwide, characterized by rare, unique, and exuberant flowers, notable for their variety of colors, sizes, and fragrances. These epiphytic, vascular, monocotyledonous, phanerogamous, and angiosperm species, belonging to various climates of tropical and temperate warm regions, conceal in their reproduction and growth a natural treasure. They are considered a thermometer of the conservation of virgin ecosystems (Mamani Sánchez et al., 2022).

In Bolivia, there are 1,350 species of orchids, 400 of which are endemic (Aguirre Villarroel et al., 2016), representing 8% of the Bolivian flora. Orchid species have been reported for the Department of Chuquisaca, belonging to 21 genera, with a growth habit of 57% epiphytic and 43% terrestrial. They grow in forests thanks to the biological diversity of ecological floors and their dry and cold climates, such as the Tucumano-Boliviano forests, the Dry Inter-Andean Valleys, and the Serrano Chaco (Vásquez, 2011).

Although orchid propagation, establishment, development, and flowering require long periods, it is possible to reduce this time and increase populations through modern biotechnological techniques such as *in vitro*, *in situ*, *ex situ*, and *ex vitro* culture. These methods also indirectly contribute to conservation by reducing extraction pressure from natural habitats and illegal trade, while providing optimal conditions and necessary nutrients for the growth and development of several genera. This enables the production of healthier cultures with specific genetic traits under controlled conditions for desired responses (Navarro, 2017).

Ex situ conservation of orchids can be greatly enhanced through *in vitro* cultivation, which provides favorable conditions for seed germination and may result in germination rates close to 100%. It also serves as an alternative method to preserve the germplasm of accessions that produce sterile seeds (Pérez-Martínez & Castañeda-Garzón, 2016). Accordingly, the objective of this research was to evaluate the performance of three native orchid (*Orchidaceae*) accessions using *in vitro* techniques in the establishment and micropropagation phases at the Biotechnology Laboratory of the BIORENA Germplasm Bank, for conservation at the Villa Carmen Yotala Research and Innovation Center.

Material and Methods

Plant material

Three accessions of orchids (*Orchidaceae*) were used. The predominant forest type is the Chiquitano Forest, a tropical dry forest characterized by its tropical or subtropical monsoon climate and an average annual temperature of 23 to 25°C. It is one of the most biodiverse ecosystems in the world and is at serious risk of disappearing. The three accessions are the only ones that were collected due to the difficulty of obtaining more individuals with fertilized capsules from native plants that were light green in color and free of physical damage. These capsules, the result of natural pollination, were collected between September and October 2023 (Figure 1) in the community of San Juan de Saguayo, Ichilo province, Santa Cruz department (17°40'00" S and 63°44'00" W) in Bolivia, at approximately 300 meters above sea level (m a.s.l.). The capsules were placed in paper envelopes, carefully sealed for transport. At the BIORENA laboratory, they were stored in a cool place and later disinfected before starting the establishment phase.

Figure 1. Orchid plants of the accessions in San Juan de Saguayo's forest.

The establishment phase was conducted using a bifactorial arrangement under a completely randomized design, with three treatments and three replications. Factor A consisted of the three orchid accessions (BAO-001, BAO-002, and BAO-003), while Factor B corresponded to different sodium hypochlorite concentrations (5%, 7%, and 10%). Ten plants were evaluated in the first phase and twenty plantlets in the second.

Establishment phase

The plant material (seeds) of the three accessions was cultivated in MS medium containing (4.43 $g*L^{-1}$), nicotinic acid (5 $ml*L^{-1}$), thiamine (1 $ml*L^{-1}$), gibberellic acid (GA_3) (5 $ml*L^{-1}$), calcium pantothenate (2 $ml*L^{-1}$), glycine (10 $ml*L^{-1}$), myo-inositol (8 $ml*L^{-1}$), sucrose (30 $g*L^{-1}$). Before sterilization, the medium was adjusted to a pH of 5.7. The medium was heated in a microwave oven for 8 min and subsequently dispensed into test tubes (3 ml per tube), immediately sealed with aluminum foil. The laminar flow chamber was disinfected with 70% alcohol, and all materials were exposed to UV light for 15 minutes. Forceps were flamed, and a closed orchid capsule was introduced for 3 minutes in the disinfectant solution (bleach at 5%, 7%, or 10%). Afterwards, it was rinsed with distilled water three times for 3 minutes each, and placed in a sterilized Petri dish. A longitudinal cut was made with a scalpel, a small amount of orchid seeds was extracted, and then the tube opening was exposed to flame and sealed with plastic film. Tubes were placed in an incubator under a 16/8 light cycle. The viability of the seeds was analyzed based on 20 days, considering protocorm development.

Multiplication phase of the three orchid accessions

For multiplication, three variants of culture media were used:

Variant 1: MS were added: sucrose (30 $g*L^{-1}$), nicotinic acid (5 $ml*L^{-1}$), thiamine (12 $ml*L^{-1}$), myo-inositol (8 $ml*L^{-1}$), glycine (10 $ml*L^{-1}$), activated charcoal (2 $g*L^{-1}$), indole-

3-acetic acid (IAA) (4 ml*L^{-1}), gibberellic acid (GA_3) (4 ml*L^{-1}), calcium pantothenate (2 ml*L^{-1}).

Variant 2: 1t was modified by adding thiamine (1 ml*L^{-1}), gibberellic acid (GA_3) (5 ml*L^{-1}) and pyridoxine (4 m*L^{-1}).

Variant 3: MS medium adding only thiamine (1 ml*L^{-1}) and gibberellic acid (GA3) (5 ml*L^{-1}).

Culture conditions

After disinfecting and sterilizing the laminar flow chamber, plantlets were extracted with forceps, selecting those with protocorms and abundant seedlings. They were transferred to different culture media and sealed with plastic film. Once sowing was completed, tubes were placed in a growth chamber equipped with LED lights programmed for 16 hours of light intensity of 18 watts 2 (At a height of 40 cm, illuminating a tray of 0.80 × 0.40 m for 16 h: Lux ≈ 3,600). The plants were evaluated during their growth and at 45 days after in vitro sowing.

Data obtained during the multiplication stage, including the count of vegetative organs, were statistically determined which means differ from one another at the 5% significance level (using ANOVA and Tukey), with the INFOSTAT statistical package. The evaluated variables were: survival percentage of plantlets, percentage of contaminated plantlets, plant height, leaf and root count.

Results

Capsules from the collected orchid accessions were also carefully examined under a microscope, once opened to verify seed integrity and quality. This analysis allowed determination of whether the seeds were complete, free of damage or deformities, and in optimal conditions for germination. Microscopic observation revealed important details about seed structure and morphology, such as size, shape, and the presence of anomalies. The results of this examination are illustrated in Figure 2, showing the appearance of the seeds, confirming their healthy state and suitability for propagation processes.

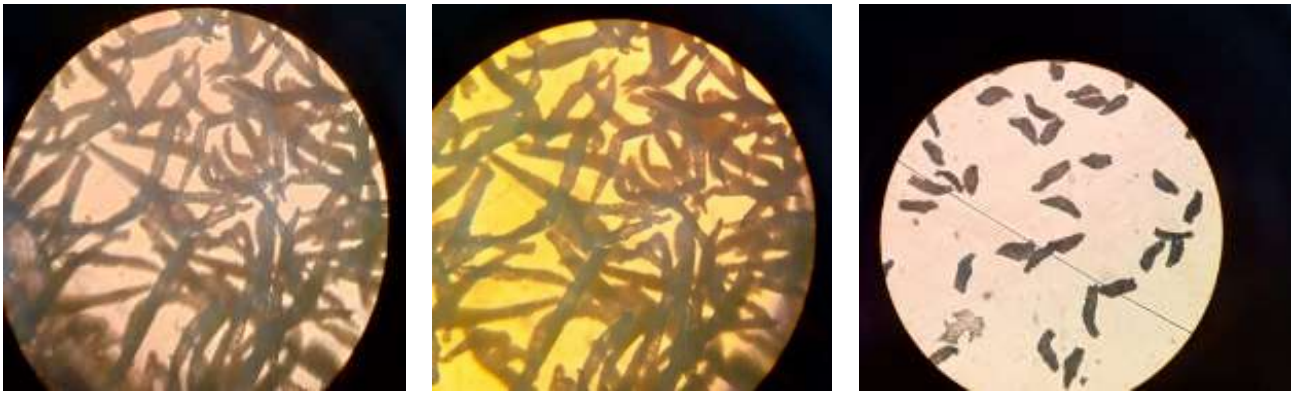

Figure 2. Microscope view of accession seeds (BAO-001, BAO-002, BAO-003) resolution 3-4 µm.

Likewise, during the sowing process in the culture media, it was observed that the seeds did not form clusters and were easily detached from the capsule. This characteristic facilitated their dispersion, since the seeds, being very small and lightweight, can disperse efficiently in the environment. This may influence both reproduction methods and cultivation techniques, allowing greater efficiency in germination and establishment processes in the culture media used for their propagation.

Establishment stage

Once cleaned and disinfected in the laboratory, the capsules were carefully opened to retrieve the seeds, which were then sown in the culture medium under sterile conditions. Then the percentage of germinated seeds at 15 days after sowing with 10% sodium hypochlorite was 100% in accession BAO-003 with no contamination, while at the same concentration accession BAO-002 showed 94.44%. At the 5% concentration, the highest result was recorded for accession BAO-001, with 83.33% of plantlets established.

Accession BAO-001 showed 16.66% contaminated plantlets in that same period; accession BAO-002 had 5.55%, and accession BAO-003 showed 0% contamination.

Seed germination and emergence under microscope observation

Testa rupture

The embryo increased in circumference and length, occupying more space, breaking the testa, and initiating germination. At 10 days, all the seeds of the three accessions germinated simultaneously.

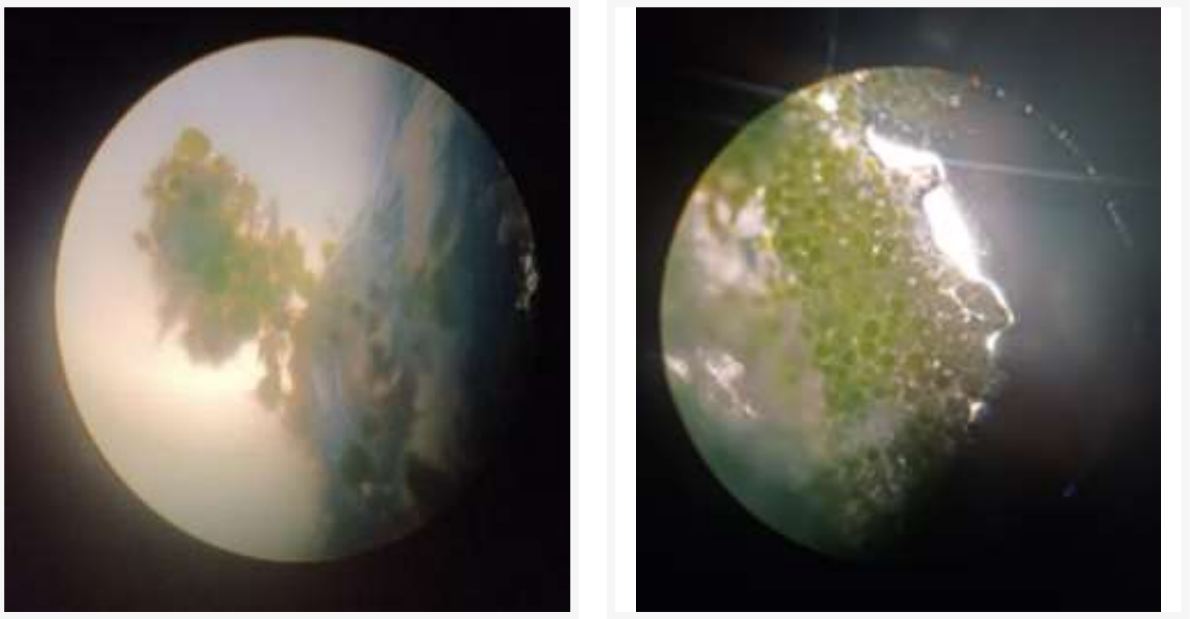

Figure 3. Microscope view of germination, resolution 3-4 µm.

The Figure 3 shows seed germination at the time of evaluation, although it was not yet possible to count the plantlets.

First leaves

The development of true leaves in the orchid accessions began approximately 30 days after germination. During this period, the plantlets started to display their first true leaves, which were clearly differentiated from the initial leaves or cotyledons (see Figure 4).

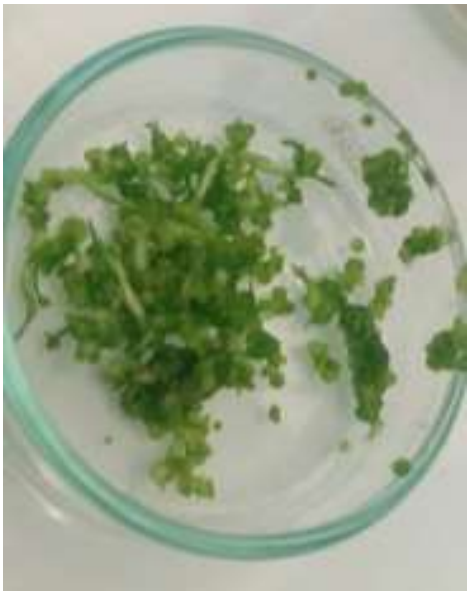

Figure 4. First leaves of in vitro plantlets (scale 35 mm).

Multiplication stage of orchid plantlets

The orchid plantlets reached a 100% survival rate during the multiplication phase across the three-culture media: T1 (MS + Activated Charcoal + Indole-3-acetic Acid), T2 (MS + Gibberellic Acid + Pyridoxine), and T3 (MS + GA_3). This demonstrates that all the media were equally effective in maintaining the viability and vitality of the plants under *in vitro* conditions. The presence of growth regulators such as IAA and GA_3, as well as the use of activated charcoal in treatment T1, created a favorable environment without causing toxic effects or physiological stress, showing that any of these treatments can be considered effective for this stage of cultivation.

With respect to contamination percentage, the three native orchid accessions multiplied in vitro survived at 100% under laboratory conditions.

The analysis of variance for plantlet height is shown in Table 1, where the results are highly significant at both the 1% and 5%

levels. This indicates that the differences observed in plantlet height among treatments or accessions are statistically relevant and not due to chance, but rather to the studied factors. The coefficient of variation (CV) obtained was 9.95%, showing stable behavior and significant differences among treatments.

Table 1
Results of the analysis of variance for the variable plantlet height (multiplication stage)

S.V.	df	SS	MS	Fc	Ft5%	Ft1%
Replication	2	27,02	13,51	4,64	3.00	4.13
Accesions (A)	2	2,67	1,34	0,46	3.00	4.13
Treatments (B)	2	32,45	16,22	9,50**	3.00	4.13
AXB	4	10,46	2,62	0,23	4.05	5.19
Experimental error	16	46,55	2,91			
Total	n-1=26	119,15				

*: significative; **: higher significative; NS: No Significative
Coefficient of Variation = 9,95 %

The comparison of the height variable among accessions was carried out using Tukey's test, where orchid plantlets at 45 days, at the 5% significance level, showed that accession BAO-003 had the highest mean height with 18.42 cm in culture medium T3 (MS + GA_3), while BAO-002 had the lowest growth with 5.74 cm.

In total, 540 plantlets were evaluated, and the number of leaves recorded during 45 days was determined under the treatments T1 = MS + IAA + AC, T2 = MS + GA3 + P, and T3= MS + GA_3.

Table 2
ANOVA summary for the leaf count (multiplication stage)

S.V.	df	SS	MS	Fc	Ft5%	Ft1%
Replication	2	64,30	32,15	1,05	3.00	4.13
Accesions (A)	2	35,85	17,93	0,58	3.00	4.13
Treatments (B)	2	582,30	291,15	9,50**	3.00	4.13
AXB	4	28,15	7,04	0,23	4.05	5.19
Experimental error	16	490,37	30,65			
Total	n-1=26	1200,96				

*: significative; **: higher significative; NS: No Significative
Coefficient of Variation=7,38 %

According to Table 2, the variance analysis of leaf count in different orchid media and accessions shows statistically significant differences at both the 1% and 5% probability levels. This indicates significant differences among treatments, confirmed by mean comparisons were performed using Tukey's test at the 5% significance level. A coefficient of variation (CV) of 7.38% indicates little dispersion in the number of leaves data.

The Tukey's test illustrations number of leaves at 45 days.

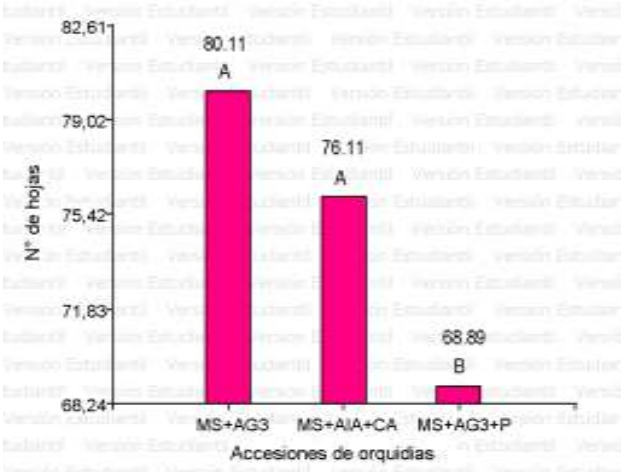

Figure 5. Number of leaves Tukey's mean comparison test.

Figure 5 shows that the accession with the highest number of leaves was BAO-003, with an average of 80 leaves, statistically similar to accession BAO-001, with an average of 76 leaves, while accession BAO-002 had the lowest number with an average of 69 leaves during the evaluated period.

The ANOVA results for root count are shown in Table 3.

Table 3
Summary of the analysis of variance for root count (multiplication stage of orchid plantlets)

S.V.	df	SS	MS	Fc	Ft5%	Ft1%
Replication	2	590,30	295,15	3,32*	3.00	4.13
Accesions (A)	2	50,30	25,15	0,28NS	3.00	4.13
Treatments (B)	2	9049,19	4524,59	50,85**	3.00	4.13
AXB	4	524,81	131,20	1,47NS	4.05	5.19
Experimental error	16	1423,70	88,98			
Total	n-1=26	11638,30				

*: significative; **: higher significative; NS: No Significative
Coefficient of Variation =32,12%

In Table 3, the variable "number of roots" is significant at both the 1% and 5% levels. In the evaluation of accessions, no significant differences were observed, while comparing culture media showed highly significant differences at 1% and 5%. However, no significant differences were found in the A×B interaction. The coefficient of variation for the variable "number of roots" was 32.12%, indicating data dispersion relative to the mean.

At 45 days Figure 6, Tukey's test during the multiplication stage showed differences among the three accessions, with accession BAO-003 presenting the highest average, with approximately 51 roots, standing out compared to the other plantlets. Accession BAO-002 had an average of approximately 32 roots, while accession BAO-001 showed the lowest mean, with approximately 6 roots, in the MS + IAA + AC medium.

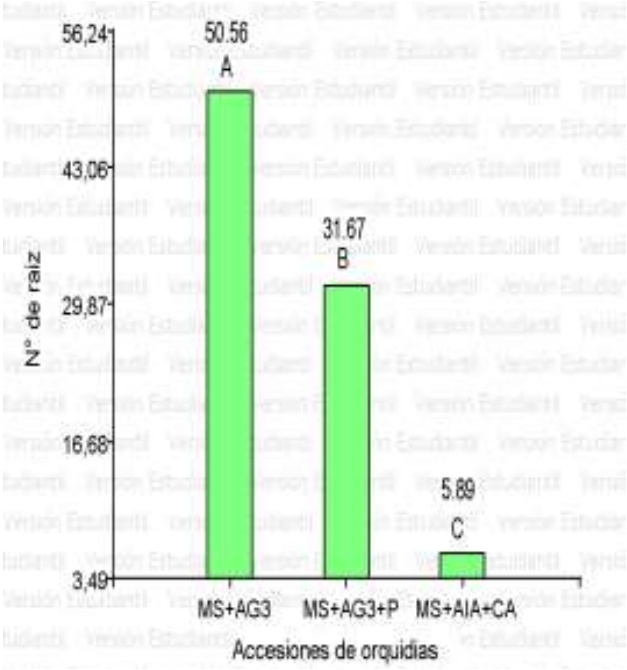

Figure 6. Mean comparison for the variable number of roots.

Discussion

The collected capsules of native orchids were fertilized under natural conditions, reaching maturity and allowing seed germination under *in vitro* conditions. (Rasmussen et al., 2015) notes that most orchids can germinate asymbiotically in a sterile culture medium where all necessary nutrients are supplied (Salgado & Peñaranda, 2019). In the establishment of orchid accessions, initial disinfection was carried out with 96% alcohol for 3 minutes, followed by three rinses. On the other hand, (Díaz-Lezcano et al., 2021) used concentrations of hypochlorite (5% and 10%) and observed

that by increasing the sodium hypochlorite concentration, the percentage of explants contaminated with fungi and bacteria was lower; however, this translated into an increase in the degree of oxidation of Musa spp. Nanicao variety explants.

The fastest and highest percentage seed germination reported (Mohanty et al., 2012) was achieved using MS medium. Seeds cultured on MS medium germinated within 3–4 weeks, whereas those grown on B5 medium required 7–8 weeks. Seedling development was also enhanced on MS medium, and the addition of plant growth regulators was not required. In this study, a temperature of 28°C was used, achieving an average of 80% seed viability after 15 days in culture medium, this behavior confirms that the germination period is longer when only MS medium is used.

The evaluation showed that seed germination occurred at 15 days, with no statistically significant differences among treatments. This indicates high uniformity in seed development and maturation, consistent with (Buendía-López et al, 2025), who described that Maximum seed germination percentage where an improved seed germination of 48% was achieved, was Knudson C media added with 0.1 mg*L-1 NAA, whereas under 3.0 mg*Ll-1, no growth was observed, results that do not match those obtained in this study.

Flores Hernández et al. (2017) reported that the *in vitro* behavior of *L. anceps* Lindl., exhibited superior performance compared with Epidendrum sp., shoots established on a perlite–tezontle as well as coconut fiber–tezontle substrates, and media with 50% salts and GA_3 no significant differences were observed in shoot length, leaf count, root count, or fresh weight relative to seedlings cultured on agar with full-strength (100%) salts and without GA_3. Media containing diluted salts without GA_3, together with alternative substrates to agar, effectively supported the in vitro growth of *L. anceps* Lindl. and *Epidendrum* sp. Consistent with these findings, native orchids in the present study performed best in the absence of GA_3.

The lowest rate contamination was 5.56%, however according to (Borjas Ventura et al., 2020), all treatments (T1, T2, T3) produced 100% healthy plants. This indicates that, although some treatments present higher initial risks, controlled conditions and aseptic measures in later stages ensure plant health, with contamination rates of up to 84.40%.

Mamani Sánchez et al. (2022) mentions that the *Masdevallia solomonii*, an endemic epiphytic orchid to the Yungas of La Paz, seeds exposed to low light intensity (1,965 µmol m^{-2} s^{-1}) exhibited a higher germination percentage (22.39%) than those exposed to high light intensity (7,992 µmol m^{-2} s^{-1}), which showed only 8.16% germination. The present study with 3,600 lux hours in germination, theoretical intensity of approximately 3,36 µm and in growth up to 45 days evaluated.

Lack of endosperm in their seeds renders orchids to depend on nutrients provided by orchid mycorrhizal fungi (OMF) for seed germination and seedling formation in the wild. (Zhao et al., 2021). However, in vitro culture, these relationships can be replaced by growth regulators, facilitating germination and propagation. At 45 days, the plantlets showed remarkable development in height (80.11 cm), number of leaves (18),

and roots (51), especially in the MS + AC + IAA, MS + P, and MS + GA_3 treatments. These results are consistent with previous studies (Ariza et al., 2018), which reported that the use of different culture media produces morphological variations in orchid plantlet growth during the multiplication stage.

In this study, GA_3 in combination with media containing lower MS concentrations (50%) significantly promoted orchid plant growth, both in leaf number and root proliferation (Pinedo-Panduro et al., 2022). Similarly, Jara et al. (2007) demonstrated that 50% and 100% MS medium were most effective for germination and *in vitro* growth in several orchid species. In this study, accession BAO-003 showed the highest leaf and root counts at 45 days, suggesting that accession response to medium and treatment may vary. In general, however, the results support the trend that lower MS concentrations and the use of GA3 improve *in vitro* growth.

Conclusions

The seeds of the three orchid accessions, disinfected with 10% sodium hypochlorite, showed 100% germination, but the survival rate of the accessions was 77.79%, and in the multiplication stage, the survival rate reached 100% at 45 days, demonstrating the effectiveness of different combinations of growth regulators in the MS medium. However, to determine the most appropriate treatment, factors such as multiplication rate, morphology, cost, and ease of medium preparation must also be evaluated.

In conclusion the MS + GA_3 medium in accession BAO-003 showed the greatest size, the highest numbers of leaves and roots.

It is recommended that the lux hours provided for 16 hours with LED tubes at an intensity between 3 to 4 µm (3.36 µm), has favored the germination and development of the evaluated vitroplants.

Author Contributions

ASRO: drafting the manuscript, tutoring, corrections, supervision and data interpretation.

BOQ: conception, data collection, and data analysis.

RAA: Contributed to drafting and corrections the manuscript.

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