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Effects of culture media composition on protocorm-like body formation and seedling mortality in *Holcoglossum amesianum*: Insights from micromorphological observations

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Abstract: *Holcoglossum amesianum* is an endangered orchid threatened by habitat destruction and overexploitation. Research on its fundamental biology, particularly the nutrient requirements for seed germination, is scarce. This study aimed to enhance the in vitro propagation efficiency of *H. amesianum* by optimising the culture medium to improve protocorm-like body (PLB) formation and seedling survival. We systematically evaluated the effects of different basal media (½MS, Hyponex, N6), organic additives (apple homogenate, AH; banana homogenate, BH), and plant growth regulators on seed germination, PLB development, and seedling mortality. The results showed that Hyponex medium supplemented with 30 g/L BH and 20 g/L AH, as well as N6 medium with 30 g/L BH, significantly promoted seed germination. However, these media combinations were associated with high subsequent PLB mortality. In contrast, half-strength Murashige and Skoog (½MS) medium supplemented with 20 g/L AH yielded a high seed germination rate and the lowest seedling mortality. These results suggest that *H. amesianum* benefits from the nutrient-rich formulation of ½MS medium supplemented with AH for successful seedling establishment in vitro. Our findings provide an optimized protocol for the conservation-oriented micropropagation of this endangered species.

Keywords: seed culture; micropropagation; plant hormones

Orchidaceae is one of the most diverse plant families, yet many species possess highly specific ecological requirements, making them vulnerable to environmental change (Zhang et al. 2018; An et al. 2021). A critical bottleneck in their life cycle is seed germination. Orchid seeds are minute and lack a functional endosperm (Lee, Yeung 2023); under natural conditions, they rely on a symbiotic relationship with mycorrhizal fungi to obtain nutrients and water for embryo development (Rasmussen, Rasmussen 2009; Yeung 2017; Utami, Hariyanto 2019; Calevo, Duffy 2023). However, the rate of successful symbiotic association in the wild is exceptionally low, leading to minimal natural recruitment (Bell et al. 2020; Phillips et al. 2020; Adit et al. 2021)

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In vitro asymbiotic seed germination offers a powerful alternative for propagating orchids, particularly for rare and endangered species (Utami et al. 2017). This technique can significantly enhance germination rates, shorten germination time, and facilitate seedling development. The nutritional needs for asymbiotic germination vary considerably among orchid genera. Species are often described as exhibiting preferences for either more nutrient-rich or more nutrient-simple media, based on their germination responses (Arditti 1984; Yeh et al. 2021). High-nutrient media (e.g., Murashige and Skoog medium) contain a full complement of macro- and micronutrients and vitamins, whereas low-nutrient media (e.g., Knudson C, Vacin & Went, Hyponex) have simpler compositions (Arditti 1984; Magrini, De Vitis 2017). Using an inappropriate medium type can result in poor outcomes: seeds with high nutrient requirements germinate poorly on low-nutrient media, while those with low requirements may germinate on rich media but subsequently suffer high protocorm mortality and stunted growth (Bhowmik, Rahman 2017; Kang et al. 2020).

The formulation of the culture medium is thus paramount. Key components include the basal salt composition, organic supplements (e.g., fruit homogenates), and plant growth regulators (PGRs) (Yao et al. 2021). Organic additives like apple or banana homogenate provide essential vitamins, amino acids, and complex sugars that can stimulate germination and growth (An et al. 2021). PGRs, particularly auxins and cytokinins, play crucial roles in regulating embryo development, cell division, and morphogenesis (Jiang et al. 2021; Zeng et al. 2022).

Holcoglossum amesianum (Rchb. f.) Christenson is an epiphytic orchid native to Southwest China, prized for its ornamental value. It is listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and is protected under Chinese law. To date, there is a paucity of information on the optimal conditions for its in vitro propagation, which hinders conservation and cultivation efforts.

Therefore, this study investigated the effects of different basal media, organic additives, and PGR combinations on the asymbiotic seed germination and subsequent development of *H. amesianum*. By determining its specific nutrient requirements and identifying an optimal culture protocol, we aim to establish a reliable method for its mass propagation, thereby contributing to the conservation of this endangered species.

MATERIAL AND METHODS

Plant material and ethical statement. Seeds of *Holcoglossum amesianum* were obtained from a mature capsule produced by a cultivated specimen maintained in the Arboretum of the College of Landscape Architecture and Horticulture, Southwest Forestry University. The mother plant is part of the university's official *ex-situ* conservation living collection (Voucher Specimen No.: SWFU1215200301). The use of seeds from this cultivated source for scientific research was authorised by the responsible authorities at Southwest Forestry University. In accordance with the provisions of the CITES and relevant national guidelines, the use of seeds derived from artificially propagated plants for non-commercial

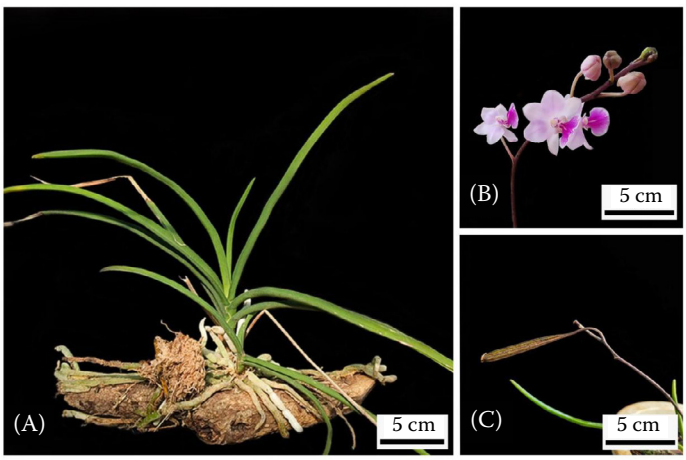

Figure 1. *Holcoglossum amesianum* (Orchidaceae): the complete morphology of the plant growing on decaying wood (A), including inflorescence and flower forms during blooming (B), as well as pod growth conditions 200 days after pollination (C)
Scale bar = 5 cm

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scientific research within the country is exempt from specific CITES trade permits. The overall research protocol was reviewed and approved by the relevant institutional ethics committee at Southwest Forestry University.

Seed sterilisation and culture initiation. Seeds were extracted by carefully scraping the interior of the capsule with a sterile scalpel. They were surface-sterilised by immersion in 75% (v/v) ethanol for 30 s, followed by treatment with a 10% (v/v) sodium hypochlorite solution containing a few drops of Tween-20 for 3 minutes. Subsequently, the seeds were rinsed four times with sterile distilled water. After filtration using a sterile mesh, approximately 200 seeds were evenly sown onto the surface of each culture medium. All experiments consisted of five replicate plates per treatment, and the entire setup was repeated three times independently.

Culture media and experimental design. All media were supplemented with 30 g/L sucrose, 1 g/L activated charcoal, and solidified with 7 g/L agar. The pH was adjusted to 5.4 before autoclaving at 121 °C for 20 minutes.

Organic additive preparation: fresh apple (*Malus domestica*) and banana (*Musa acuminata*) were peeled, homogenised in distilled water at a ratio of 1 : 1 (w/v), and filtered through two layers of cheesecloth. The resulting homogenates (apple homogenate (AH) and banana homogenate (BH), respectively) were added to the media prior to autoclaving.

Preliminary experiments (MA and MB series): These experiments aimed to screen basic conditions.

MA: Quarter-strength MS (¼MS) medium supplemented with two combinations of 6-benzylaminopurine (6-BA) and α-naphthaleneacetic acid (NAA) (0.5 mg/L each or 1.0 mg/L each), tested at pH 5.0 and 5.4.

MB: Two basal media (Hyponex N-P-K 7 : 6 : 19 and half-strength MS, ½MS) were tested, both containing 0.5 mg/L each of 6-BA and NAA, and with or without 20 g/L AH.

Optimisation experiment (MD series): A three-factor experimental design was used to evaluate the combined effects of basal medium, BH, and AH. Factors and levels were: basal medium (Hyponex, ½MS, N6); BH (0, 30, 60 g/L); AH (0, 20, 40 g/L). All MD media contained 0.5 mg/L each of 6-BA and NAA. Factors and levels were: basal medium (Hyponex, ½MS, N6); BH (0, 30, 60 g/L); AH (0, 20, 40 g/L). All MD media contained 0.5 mg/L each of 6-BA and NAA.

Data collection and microscopic observation. Seed germination, defined as the emergence of a green protocorm, was monitored every two days. Germination time and counts were recorded. At 20 days after sowing, average-sized protocorms from each treatment were sampled (n = 10) to measure embryo diameter under an OLYMPUS light microscope (Olympus Corporation, Tokyo, Japan). Seedling formation (development of the first leaf) and mortality (bleaching and collapse of the protocorm/seedling) were recorded up to 40 days.

The following parameters were calculated:

Germination percentage (%) = (No. of germinated seeds/total seeds sown) × 100

Seedling formation rate (%) = (No. of seeds forming seedlings by day 40/total seeds sown) × 100

Mortality rate (%) = (No. of germinated seeds that died by day 40/total number of germinated seeds) × 100

Statistical analysis. The experiment followed a completely randomised design. For statistical analysis, the mean value from each independent experimental run (n = 3) was used as the unit of replication. Data were analysed using one-way ANOVA in SPSS software (version 26). Treatment means were compared using Duncan's multiple range test at a significance level of $P \leq 0.05$. Data are presented as mean ± standard deviation (SD) of the three independent replications.

RESULTS

Effects of hormones and organic additives on early germination. The preliminary MA series, testing hormone concentrations and pH in ¼MS medium, resulted in low germination rates (1.07 to 2.37%) and slow germination (initiating at 20 days) (Table 1). The MB series, which introduced organic additives, showed marked improvement. Germination commenced earlier (15 days) and rates increased significantly. The combination of ½MS medium, 0.5 mg/L each of 6-BA and NAA, and 20 g/L AH (treatment MB2) yielded the highest germination rate (19.93 ± 1.35%) and the highest subsequent seedling formation rate (16.97 ± 1.15%), with a relatively low mortality rate (14.87 ± 2.16%).

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Table 1. Effects of different concentrations of plant growth regulators (PGRs) and organic additives on *Holcoglossum amesianum* seed germination and early development

Code	Treatment				Germination time (days)	Germination percentage	Seedling formation rate	Mortality rate
	BM	PGRs (mg/L)	AH (g/L)	pH		(%) (mean ± SD)		
MA1	¼MS	0.5	–	5.0	20	1.57 ± 0.45^{c}	1.00 ± 0.17^{c}	36.47 ± 5.89^{b}
MA2	¼MS	0.5	–	5.4	20	2.37 ± 0.49^{c}	1.13 ± 0.25^{c}	52.47 ± 2.83^{b}
MA3	¼MS	1	–	5.0	20	1.07 ± 0.32^{c}	0.87 ± 0.15^{c}	27.86 ± 7.49^{c}
MA4	¼MS	1	–	5.4	20	2.13 ± 0.45^{c}	2.17 ± 0.20^{b}	4.15 ± 3.88^{c}
MB1	Hy	0.5	20	5.4	15	10.30 ± 1.03^{b}	3.10 ± 0.26^{b}	69.80 ± 1.44^{a}
MB2	½MS	0.5	20	5.4	15	19.93 ± 1.35^{a}	16.97 ± 1.15^{a}	14.87 ± 2.16^{c}

BM –basic medium; PGRs – 6-benzylaminopurine (6-BA) and α-naphthaleneacetic acid (NAA); AH – apple homogenate; ½MS – half-strength Murashige and Skoog medium; ¼MS – quarter-strength Murashige and Skoog medium; Hy – Hyponex medium; the values represent the means (± SD) of three independent assessments; within each column, means followed by different lowercase letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$)

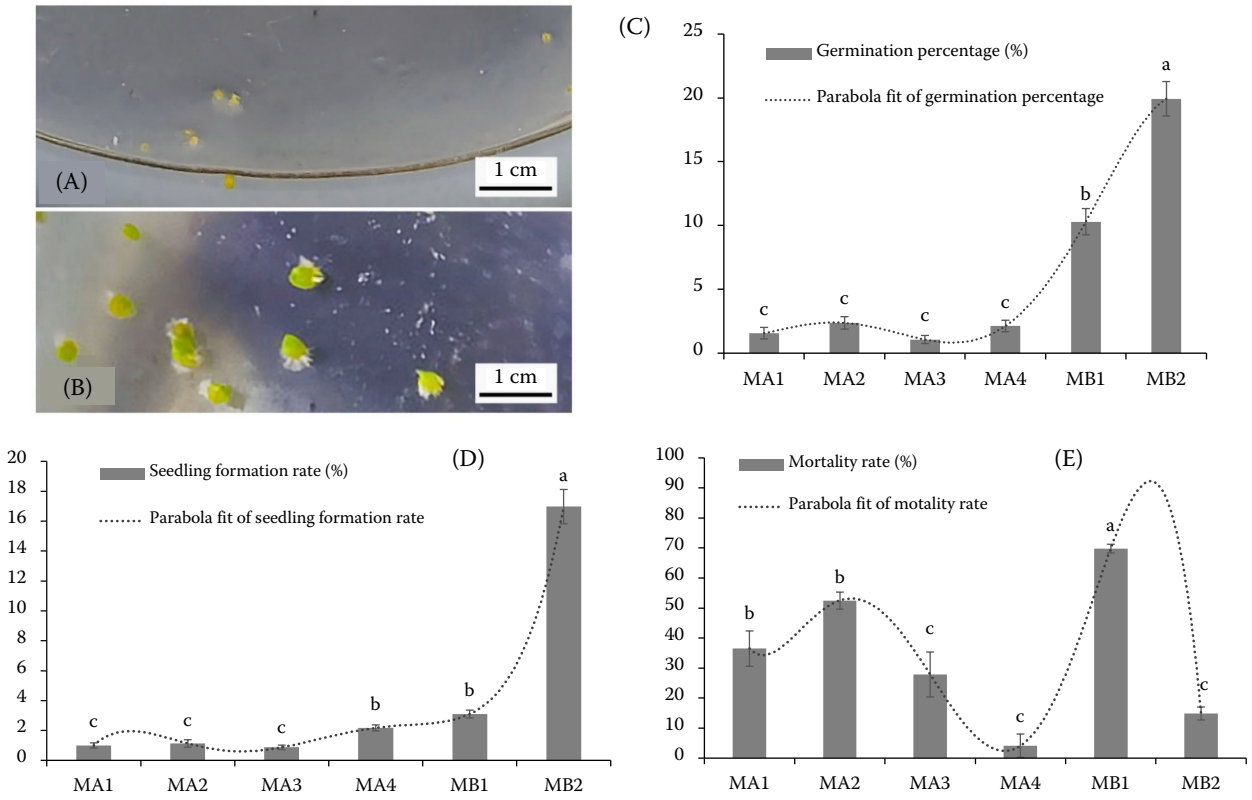

Figure 2. Seed germination and early development of *Holcoglossum amesianum* on MB2 medium (½MS + 0.5 mg/L 6-BA & NAA + 20 g/L AH): (A) protocorms at 20 days after sowing (scale bar = 1 mm, (B) seedlings at 40 days after sowing (scale bar = 1 cm), (C) germination percentage across treatments, (D) seedling formation rate across treatments, (E) mortality rate of germinated seeds across treatments

½MS – half-strength Murashige and Skoog medium; 6-BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; AH – apple homogenate; (C–E) – data are presented as mean ± SD (n = 3); bars topped with different letters indicate significant differences ($P \leq 0.05$) based on Duncan's test

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Table 2. Effects of basal medium, banana homogenate (BH), and apple homogenate (AH) on seed germination and early protocorm size

Code	Treatment			Germination time (days)	Germination percentage (%) (mean ± SD)	Protocorm diameter at 20 days (mm)
	BM	BH	AH			
		(g/L)				
MD1	Hy	0	0	10	10.37 ± 0.32^{d}	2.00
MD2	½MS	0	20	10	20.90 ± 0.36^{b}	2.10
MD3	N6	0	40	10	3.03 ± 0.46^{f}	0.95
MD4	Hy	30	20	10	29.00 ± 1.30^{a}	1.40
MD5	½MS	30	40	10	14.50 ± 0.5^{c}	1.50
MD6	N6	30	0	10	29.23 ± 0.49^{a}	0.95
MD7	Hy	60	40	10	7.33 ± 0.30^{e}	1.15
MD8	½MS	60	0	10	10.00 ± 0.72^{d}	0.50
MD9	N6	60	20	10	2.03 ± 0.56^{f}	0.50

BM –basic medium; Hy – Hyponex medium; ½MS – half-strength Murashige and Skoog medium; the values represent the means of three independent assessments; within each column, values followed by different lowercase letters are significantly different according to Duncan's multiple range test ($P \leq 0.05$)

In contrast, the same PGRs with Hyponex and AH (MB1) led to lower germination and a very high mortality rate (69.80 ± 1.44%) (Table 1, Figure 2).

Optimization of culture medium via orthogonal design. The orthogonal experiment (MD1–MD9) revealed significant interactions among basal medium, BH, and AH (Table 2). The highest germination rates (approximately 29%) were achieved with Hyponex + 30 g/L BH + 20 g/L AH (MD4) and N6 + 30 g/L BH (MD6). However, protocorm size at 20 days varied; the largest protocorms (2.00 to 2.10 mm in diameter) were observed in media without BH (MD1, MD2), while those in media with high BH or N6 basal medium remained smaller (0.50–1.50 mm) (Table 2, Figure 3A).

Crucially, the media that promoted the highest germination (MD4, MD6) did not support the highest seedling formation. After 30–40 days, significant mortality occurred (Figure 3C). The subsequent development revealed stark contrasts (Table 3, Figure 4). Treatment MD2 (½MS + 20 g/L AH) consistently performed best, yielding the highest seedling formation rate (16.40 ± 0.85%) and the lowest mortality rate (21.47 ± 5.39%). In contrast, treatments with high concentrations of BH (e.g., MD5: ½MS + 30 g/L BH + 40 g/L AH; MD7: Hyponex + 60 g/L BH + 40 g/L AH) resulted in seedling formation rates below 2.5% and mortality rates exceeding 70%. N6-based media generally performed poorly in supporting seedling development.

Optimisation experiments effect on seedling formation and mortality rates. After 30 days, the formation of buds at the apex of the protocorm could be observed with the naked eye, which is a significant development indicating progression towards the next growth stage. However, alongside this positive development, a phenomenon of reduced germination and seedling formation was also observed (Figure 3C). In the subsequent growth of seedlings, MD2 had the best growth and the most developed root system (Figure 5).

Among all treatment groups, the MD2 group demonstrated remarkable results in terms of both a high germination rate and a high seedling formation rate. With a seedling formation rate of 16.40% (Figure 4). On the other hand, while both the MD4 and MD6 groups exhibited optimal germination rates among all treatment groups, their seedling formation rates were disappointingly low at around 8.50%. This indicates that while these media supported high initial germination rates, they were insufficient to support sustained development through to seedling formation. The MD9 treatment group exhibited a significantly reduced germination rate compared to other groups. With a mere 1.23% seedling formation rate, it suggests that this specific combination of culture medium and fruit pulps may not be conducive for promoting seedling growth. The MD2 group,

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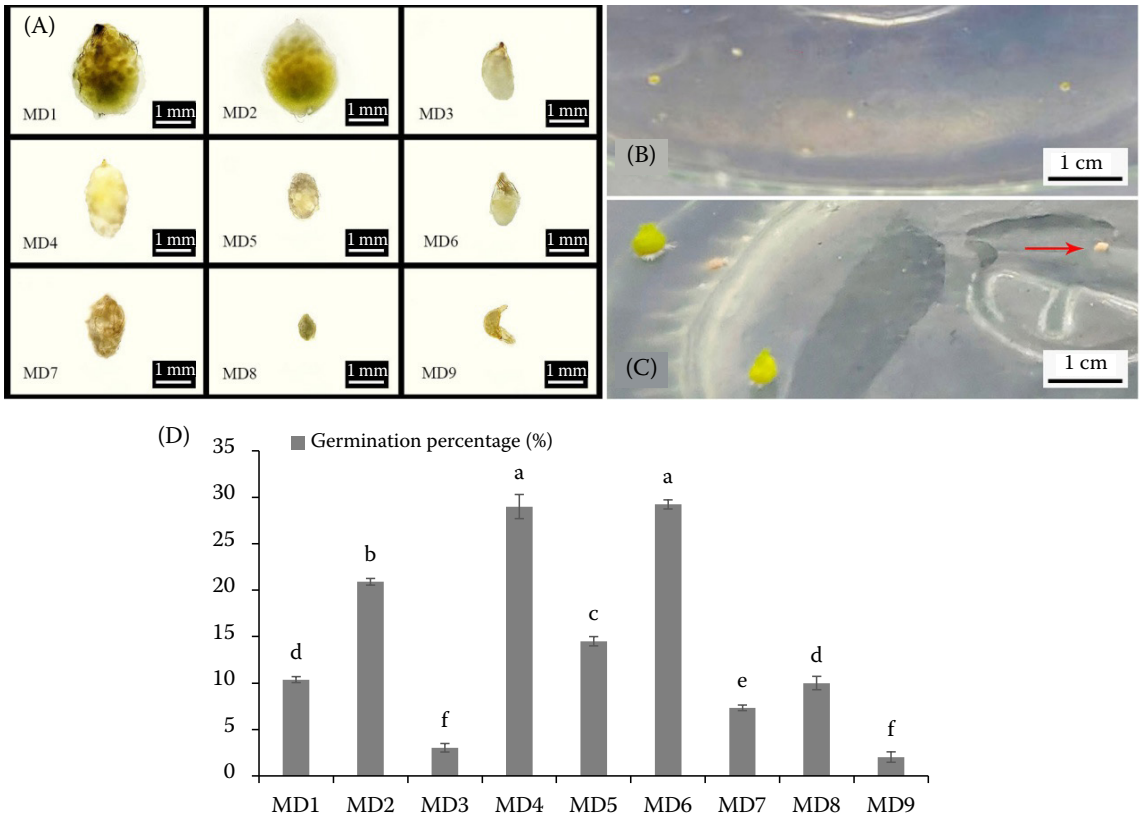

Figure 3. Representative images and data showing the effects of culture media on protocorm development and viability: (A) protocorms from different treatments (MD1–MD9) at 20 days after sowing (scale bar = 1 mm), (B) protocorm development at 20 days and (C) protocorm mortality (indicated by arrows) at 40 days under different culture conditions (scale bar = 1 cm), (D) germination percentage across treatments

Data are shown as mean ± SD (n = 3); bars topped with different letters indicate significant differences ($P \leq 0.05$) based on Duncan's test

Table 3. Effects of basal medium, banana homogenate (BH), and apple homogenate (AH) on seedling formation and mortality

Code	Treatment			Seedling formation time (days)	Seedling formation rate	Mortality rates
	BM	BH	AH			
		(g/L)			(%) (mean ± SD)	
MD1	Hy	0	0	30	1.93 ± 0.49^{d}	81.24 ± 5.41^{ab}
MD2	½MS	0	20	30	16.40 ± 0.85^{a}	21.47 ± 5.39^{e}
MD3	N6	0	40	30	2.07 ± 0.21^{d}	30.91 ± 11.22^{de}
MD4	Hy	30	20	30	8.50 ± 0.26^{b}	70.62 ± 2.28^{c}
MD5	½MS	30	40	30	2.23 ± 0.31^{d}	84.54 ± 2.61^{a}
MD6	N6	30	0	30	8.77 ± 1.18^{b}	69.99 ± 2.50^{c}
MD7	Hy	60	40	30	2.03 ± 0.32^{d}	72.11 ± 5.63^{bc}
MD8	½MS	60	0	30	3.77 ± 0.28^{c}	62.06 ± 5.78^{c}
MD9	N6	60	20	30	1.23 ± 0.45^{d}	39.05 ± 5.54^{d}

BM –basic medium; Hy – Hyponex medium; ½MS – half-strength Murashige and Skoog medium; the values represent the means (± SD) of three independent assessments; the mean values in a column followed by the same letter do not differ significantly at $P \leq 0.05$

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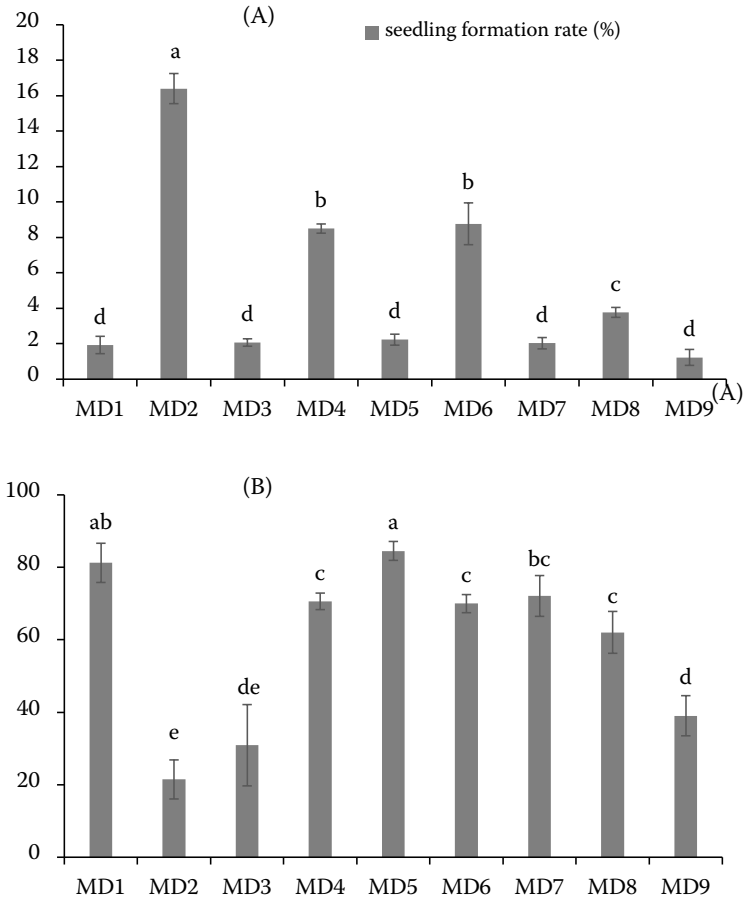

Figure 4. Effects of basal medium, banana homogenate (BH), and apple homogenate (AH) on seedling formation and mortality: (A) seedling formation rate across treatments, (B) mortality rate across treatments
Data are shown as mean ± SD (n = 3); bars topped with different letters indicate significant differences ($P \leq 0.05$) based on Duncan's test0

with a death rate of 21.47%, demonstrated the lowest level of seed mortality among all of the studied treatment groups. In contrast, the MD5 group exhibited the highest death rate at 84.54%. This suggests that substances present in BH may stimulate rapid growth and also contribute to premature demise in seeds.

DISCUSSION

The formulation of the culture medium is paramount for the asymbiotic propagation of orchids, with requirements varying significantly among species (Jolman et al. 2022). Contrary to some reports that certain orchids, like many *Paphiope-*

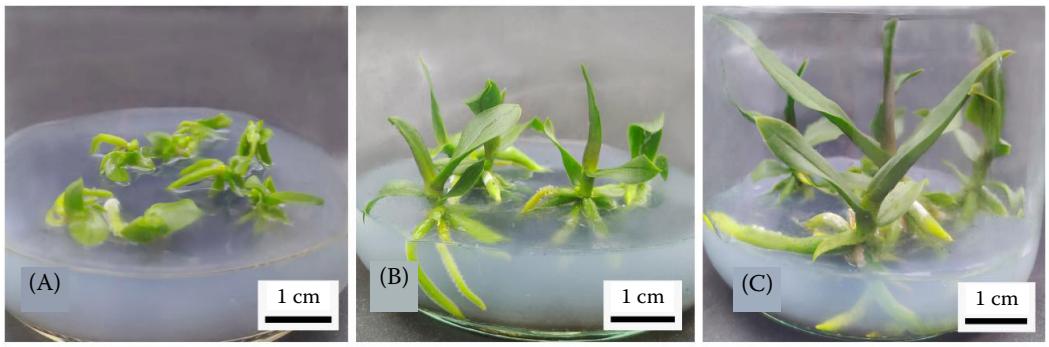

Figure 5. Representative seedling morphology at different culture stages on MD2 medium: (A) seedlings at 60 days after sowing, (B) seedlings at 70 days, (C) seedlings at 100 days
Scale bar = 1 cm

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dilum varieties, prefer low-nutrient media (Kang et al. 2020), our results suggest that *Holcoglossum amesianum* benefits from a relatively nutrient-rich environment under the tested conditions. The very low germination on ¼MS medium and the superior seedling establishment on ½MS medium compared to simpler media like Hyponex (MB1 vs. MB2) align it with other high-nutrient demanders (Bhowmik, Rahman 2017). This interspecific variation underlines the necessity of empirical optimisation for each taxon, as generalised media formulas often fail to meet specific physiological needs during critical developmental windows, such as the transition from heterotrophic to autotrophic growth.

Organic additives played a critical but nuanced role (Park et al. 2023; Guo et al. 2024; Hwang et al. 2024). While BH is rich in essential elements, vitamins, and amino acids (Kaur, Bhutani 2016), our data indicate that its effect is concentration-dependent. High concentrations of BH (≥ 30 g/L) were associated with severe mortality (e.g., MD5, MD7), likely due to osmotic stress or the introduction of growth-inhibitory compounds such as phenolics, a phenomenon noted in other plant tissue culture systems (Prando et al. 2014). The stark contrast between the promotion of germination and the induction of mortality suggests that BH may contain both stimulatory and inhibitory factors, with the net effect determined by concentration and interaction with the basal salts. In contrast, AH at 20 g/L provided beneficial organic supplements without apparent negative effects, effectively supporting sustained growth when combined with the balanced salts of ½MS medium.

The success of the PGR combination (0.5 mg/L NAA and 6-BA) in our optimal medium underscores the importance of a balanced auxin-cytokinin ratio for orchestrating cell division and morphogenesis in germinating orchid seeds (Hossain et al. 2010; Lal, Singh 2020; Zeng et al. 2022). It is noteworthy that this PGR ratio was ineffective in a suboptimal basal medium (¼MS, MA series), highlighting that an appropriate nutritional foundation is a prerequisite for phytohormones to exert their positive regulatory effects. This interdependence between nutrients and PGRs is crucial for optimising protocols for recalcitrant species like *H. amesianum*. Furthermore, the high mortality observed in high-BH media, despite the presence of PGRs, suggests that suboptimal culture conditions can override the positive effects of hormonal balance, ultimately leading to developmental failure.

CONCLUSION

In conclusion, this study identifies the specific nutrient requirements for the in vitro propagation of the endangered orchid *Holcoglossum amesianum*. We found that half-strength Murashige and Skoog (½MS) medium supplemented with 20 g/L apple homogenate and a combination of 0.5 mg/L 6-BA and NAA is optimal, supporting both efficient seed germination (20.9%) and the highest seedling formation rate (16.4%) with minimal mortality (21.5%). While other media (Hyponex with BH and AH, N6 with BH) promoted high initial germination, they led to unsustainable growth and high seedling loss. These results suggest that *H. amesianum* benefits from a relatively nutrient-rich environment for successful seedling establishment under asymbiotic conditions. The optimised protocol established here provides an effective method for the in vitro propagation of *H. amesianum*, contributing to ex situ conservation efforts and supporting future reintroduction programs for this endangered species.

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