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In vitro seed germination, differentiation and phytochemical screening of the medicinal orchid *Acampe ochracea* of Bangladesh

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Abstract
Acampe ochracea (Lindl.) Hochr. is a medicinally significant epiphytic orchid indigenous to Bangladesh, currently threatened by habitat degradation and overharvesting. This study establishes a reproducible protocol for asymbiotic seed germination and comparative phytochemical profiling to facilitate *ex situ* conservation and pharmaceutical utilization. Immature seeds were cultured on four basal media (MS, PM, MVW and KC) supplemented with (0.5 mg/l BAP and 0.5 mg/l NAA) or without plant growth regulators (PGRs). PM (Phytamax) medium fortified with PGRs proved superior, yielding the highest germination rate (73.34%) and the earliest protocorm initiation (9.43 ± 0.25 weeks). Complete plantlet development, characterized by healthy root and shoot differentiation, was achieved within 24.33 weeks. Following acclimatization on a coir, charcoal and brick enriched substrate, seedlings exhibited an 82% survival rate. Furthermore, qualitative phytochemical screening revealed that *in vitro* derived tissues synthesized comparable or superior levels of secondary metabolites specifically alkaloids and saponins compared to wild counterparts. These findings confirm the efficacy of the protocol for mass propagation and suggest that *in vitro* culture conditions may enhance the biosynthesis of bioactive compounds, offering a sustainable alternative to wild harvesting for medicinal applications.

Keywords: *Acampe ochracea*, asymbiotic germination, phytamax medium, phytochemical screening, medicinal orchid

Introduction

Orchids represent one of the most diverse plant families globally, with more than 26,000 species exhibiting extraordinary ecological, medicinal and ornamental values [1]. *Acampe ochracea* (Lindl.) Hochr., a widespread epiphytic orchid in South and Southeast Asia, holds considerable medicinal importance. Extracts of *Acampe* species are traditionally used to treat inflammation, liver disorders and microbial infections and phytochemical reports reveal the presence of phenolics, alkaloids, terpenoids and antioxidant compounds [2-3].

Despite its medicinal potential, *A. ochracea* faces propagation challenges. Orchid seeds lack endosperm and depend on fungal associations for germination, making natural regeneration slow and unpredictable [4]. Overharvesting, habitat degradation and epiphytic dependence further threaten wild populations in Bangladesh [5].

Tissue culture provides a practical solution for orchid conservation and mass propagation. *in vitro* seed germination avoids mycorrhizal dependence and supports rapid, uniform seedling production [6] and successfully applied to several medicinal orchids such as *Dendrobium*, *Rhynchostylis* and *Cymbidium* [7-8].

Phytochemical profiling of *in vitro* grown orchids also offers advantages. Controlled conditions often enhance biosynthesis of secondary metabolites and medicinal properties [9-10]. However, no comprehensive study has reported the *in vitro* germination, differentiation and phytochemical characteristics of *A. ochracea* in Bangladesh. Therefore, this study aimed to establish an efficient *in vitro* seed germination protocol, describe the developmental differentiation patterns, perform phytochemical screening of *in vitro* raised plants and optimize hardening and acclimatization methods to support successful *ex situ* conservation.

Materials and Methods

Seed collection and capsule maturity

Immature green capsules of *A. ochracea* were collected from naturally growing orchid populations in the Bandarban, Chittagong Hill Tracts, Bangladesh. Capsules aged 90-110 days post pollination were selected, as this stage gives the highest viability.

Surface sterilization protocol

Surface sterilization was carried out by first washing the capsules under running tap water for 20 minutes, followed by a 2% (v/v) Tween-20 wash for 5 minutes and treatment with 0.1% (w/v) Bavistin solution for 10 minutes. The capsules were then rinsed in 70% ethanol for 30 seconds and subsequently immersed in 0.1% $HgCl_2$ for 5 minutes. Finally, they were washed three times with autoclaved distilled water, after which the seeds were aseptically scooped out and inoculated onto the culture media under laminar airflow conditions.

Culture media and conditions

Four basal media, prepared with or without plant growth regulators (PGRs), were used to assess germination efficiency: MS [11] supplemented with 3% sucrose; PM [12] with 2% sucrose; MVW [13] with 2% sucrose and KC [14] with 2% sucrose. All formulations were solidified using 0.8% agar (Fluka), and the pH was carefully adjusted 5.8 for MS, 5.4 for PM and MVW and 5.0 for KC prior to sterilization by autoclaving at 121 °C for 20 minutes.

Developmental stages recorded

The developmental progression of cultured seeds was monitored at regular intervals and categorized into distinct stages, including initial seed swelling indicating early germination, formation of protocorms, differentiation of protocorms marked by the appearance of leaf primordia,

development of seedlings reaching the two-leaf stage, subsequent shoot multiplication and finally root induction leading to complete plantlet formation. Cultures were incubated at 25 ± 2 °C, 2500-3000 lux Light intensity under a 14/10-hour light/dark photoperiod.

Hardening and acclimatization

Well rooted seedlings measuring 3-5 cm in height were carefully transferred to plastic trays containing a substrate mixture of 40% coconut coir, 30% charcoal pieces and 30% broken brick chips. To ensure successful acclimatization, humidity was maintained at 80-90% for the first two weeks by covering the trays with transparent lids. The seedlings were gradually exposed to greenhouse conditions over a period of four weeks and the survival percentage was recorded after 60 days.

Phytochemical Screening

Sample Preparation

Fresh *in vitro* grown plantlets were collected, oven dried at 40°C, finely powdered and extracted using 80% methanol. The extracts were filtered and concentrated for subsequent qualitative analyses.

Qualitative tests

Phytochemical screening was conducted on both naturally grown and *in vitro* developed plantlets of *A. ochracea* to determine key secondary metabolites, including alkaloids, phlobatannins, flavonoids, terpenoids, steroids, glycosides, quinine, coumarin, anthraquinone, saponins and tannins. Plant materials (leaf, stem and root of natural plants; whole parts of *in vitro* plantlets) were collected from Bandarban, CHT, Bangladesh and analyzed within 6-24 hours to prevent degradation of active compounds. Alkaloid detecting reagents; Dragendorff's, Hager's, Mayer's, Wagner's and tannic acid were prepared following standard protocols [15]. Qualitative spot tests for alkaloids followed the methods of Webb [16], later modified by Aplin and Canon [17], where acidic plant extracts were reacted with specific reagents and the resulting precipitate intensity recorded using a "–" to "+++" scale. Additional secondary metabolite assays were performed using established phytochemical protocols: phlobatannins [18], flavonoids [18], saponins [19], tannins [20], terpenoids and steroids [21], glycosides [20], anthraquinones, quinine and coumarins [22]. All tests were carried out using crude extracts prepared in aqueous or methanolic solutions according to the respective procedures.

Results

The seeds of *Acampe ochracea* (Lindl.) Hochr, an epiphytic indigenous orchid was aseptically germinated on four media *viz.* KC [14]; MS [11]; PM [12] and MVW [13] with or without PGRs (Table -1, Figure 1-2). The highest rate of seed germination on culture vessel was observed on PGRs (0.5 mg/l BAP + 0.5mg/l NAA) supplemented PM medium (73.34%) followed by PGRs fortified MS (66.67%), KC (46.67%) and MVW (33.34%) respectively. Whereas, the lowest percentage (26.67%) was recorded on PGRs free MVW medium. The initiation of seed germination was obtained within the lowest time (9.43 ± 0.25 weeks) on PGRs fortified PM medium, but the maximum time required (16.20 ± 0.32 weeks) on PGRs free MVW medium. For the development of protocorms, differentiation of first leaf premordia, differentiation of first root premordia and development of seedlings required minimum time on PGRs supplemented PM medium (9.43 ± 0.25, 13.03 ± 0.03, 17.07 ± 0.32, 21.10 ± 0.26 and 24.33 ± 0.27 weeks) respectively. Whereas, the utmost time requisite for the development of protocorms, differentiation of first leaf primordial, differentiation of first root primordial and development of seedlings was recorded on PGRs free MVW medium within (16.20 ± 0.32, 20.20 ± 0.35, 24.20 ± 0.29, 29.60 ± 0.34, 34.20 ± 0.30 weeks) respectively. PM medium is better for this orchid seed's germination and differentiation than other three MS, KC and MVW media accordingly. The coir-charcoal-brick substrate exhibited 82% survival. Seedlings produced new leaves within 3-4 weeks of acclimatization.

In this study, both *in vitro* developed and naturally grown plant parts were subjected to qualitative phytochemical screening for key secondary metabolites, which are organic compounds not directly involved in growth or reproduction but play crucial roles in plant defense against microorganisms, insects and herbivores [23]. These metabolites are also valuable to humans as medicines, flavorings, and recreational compounds. The secondary metabolites evaluated included alkaloids, flavonoids, phlobatannins, steroids, terpenoids, coumarins, quinines and glycosides. Alkaloids were assessed using five reagents: Dragendorff's, Mayer's, Hager's, Wagner's and Tannic acid. The relative presence of each metabolite in plant extracts was indicated using a graded scale: '+' (low), '++' (moderate), and '+++' (high), while absence was marked as '-'. Color intensity served as the analytical indicator.

In the qualitative alkaloid analysis, Wagner's (W) reagent showed higher responses in *in vitro* developed plants compared to naturally grown ones, indicating a higher alkaloid concentration in the *in vitro* plants and highlighting differences between the two sources of *A. ochracea* (Table 2). Conversely, Mayer's (M) reagent indicated lower alkaloid levels in naturally grown plants, further suggesting that *in vitro* developed plants possess superior alkaloid content. Tannic acid reagent results showed lower alkaloid content in *in vitro* plants compared to naturally grown plants. However, Dragendorff's (D) and Hager's (H) reagents revealed similar alkaloid concentrations in both *in vitro* and naturally grown plants. Overall, only minor differences were observed between *in vitro* developed and naturally grown plants, with *in vitro* plants generally exhibiting higher alkaloid levels.

The comparative qualitative analysis of secondary metabolites in *in vitro* developed and naturally grown *A. ochracea* plant parts (Table 3). Terpenoids and quinines were present at high concentrations in both *in vitro* and naturally grown leaves, stems and roots, indicating no significant difference between the two sources. In the saponin test, naturally grown leaves, stems and roots showed variable low to moderate levels (+, -, ++), whereas *in vitro* developed plantlets showed '+++', suggesting higher saponin content *in vitro*. Glycosides and anthraquinones were absent ('-') in both naturally grown leaves and *in vitro* plantlets, although anthraquinone was present at low levels (+) in the roots and stems of naturally grown plants. Phlobatannins were present in similar concentrations in *in vitro* and naturally grown stems and roots ('++'), though naturally grown leaves lacked this metabolite. Steroids were detected in both types: naturally grown leaves (+++), roots (++), stems (+) and *in vitro* developed plantlets (++) indicating overall presence.

Coumarins were present in both, with *in vitro* plantlets showing moderate levels ('++') and naturally grown roots and stems showing higher concentrations (+++). For flavonoids, *in vitro* plantlets exhibited moderate levels ('++'), comparable to naturally grown stems, while naturally grown roots had higher levels (+++) and leaves lower (+), suggesting slightly higher flavonoid accumulation in specific natural organs compared to *in vitro* plantlets. Overall, *in vitro* developed plantlets generally exhibited comparable or higher concentrations of key secondary metabolites than naturally grown plants.

Table 1: Effect of KC, MS, PM and MVW media with or without PGRs on *in vitro* seed germination, differentiation and seedlings development of A. ochracea (Lindl.) Hochr

Medium	Strength of medium	Time taken in weeks					% of culture vessel germinated	Remarks
		Initiation of germination (Mean ± SE)	Development of protocorms (Mean ± SE)	Differentiation of 1st leaf primordia (Mean ± SE)	Differentiation of 1st root primordia (Mean ± SE)	Development of seedlings (Mean ± SE)		
KC	Full without PGRs	15.07 ± 0.27	19.07 ± 0.30	24.40 ± 0.31	28.17 ± 0.35	33.13 ± 0.31	33.34	+
	Full with PGRs	12.40 ± 0.31	16.40 ± 0.26	20.10 ± 0.36	25.07 ± 0.34	30.20 ± 0.29	46.67	+
MS	Full without PGRs	12.07 ± 0.32	17.13 ± 0.28	22.20 ± 0.30	26.07 ± 0.32	31.10 ± 0.32	53.34	++
	Full with PGRs	10.20 ± 0.30	14.27 ± 0.33	19.03 ± 0.33	23.10 ± 0.29	28.23 ± 0.34	66.67	++
PM	Full without PGRs	11.30 ± 0.29	16.27 ± 0.27	21.27 ± 0.28	24.37 ± 0.26	29.13 ± 0.29	60.00	++
	Full with PGRs	9.43 ± 0.25	13.03 ± 0.30	17.07 ± 0.32	21.10 ± 0.26	24.33 ± 0.27	73.34	++
MVW	Full without PGRs	16.20 ± 0.32	20.20 ± 0.35	24.20 ± 0.29	29.60 ± 0.34	34.20 ± 0.30	26.67	+
	Full with PGRs	13.13 ± 0.26	17.07 ± 0.28	21.37 ± 0.24	26.37 ± 0.24	30.07 ± 0.28	33.34	+

PGRs (0.5mg/l BAP + 0.5mg/l NAA); + = Minimum germination (0% ≤ + ≤ 49%), ++ = Medium germination (50% ≤ ++ ≤ 74%), +++ = Maximum germination (75% ≤ +++ ≤ 100%). Values represent mean ± SE of each experiment consist of 12 replicates.

Fig 1: Germination of *A. ochracea* seeds on PM + 0.5 mg/l BAP + 0.5 mg/l NAA

Fig 2: Plantlet of *A. ochracea* developed on MS + 0.5 mg/l BAP + 0.5 mg/l NAA

Table 2: Qualitative test for alkaloids of *A. ochracea* (Natural and *in vitro* plant parts)

Plant sample used	Plant parts	Qualitative estimation of alkaloids by different reagents				
		D	H	M	W	T
Natural plant parts	Leaf	++	+++	+++	++	+++
	Root	++	++	+++	++	+++
	Stem	+++	+++	++	++	+++
In vitro plant parts	Callus	++	+++	+++	+++	++
	Shoot bud	++	++	+++	+++	+++
	SPSS	+++	++	+++	++	+++

Notes: Name of the reagents, D-Dragendroff's reagent, H-Hager's reagent, M-Mayer's reagent, W-Wagner's reagent and T- Tannic acid reagent.

Table 3: Qualitative test for secondary metabolites of *A. ochracea* (Natural and *in vitro* plant parts)

Plant parts used	Plant parts	Secondary metabolites (% of coloration)									
		Ter.	Qui.	Str.	Cou.	Flv.	Sap.	Tann.	Phl.	Gly.	Anthro.
Natural plant parts	Leaf	+++	+++	+++	++	++	+	+	-	-	-
	Root	+++	++	++	+++	+++	-	+	++	-	+
	Stem	+++	+++	+	+++	++	++	++	++	-	+
In vitro plant parts	Plantlets	+++	++	++	+	++	+++	+++	++	-	-

Notes: Ter.= Terpenoids, Qui. = Quinine, Str.= Steroids, Cou. = Coumarin, Flv. = Flavonoids, Sap. =Saponine, Tann. = Tannin, Phl. = Phlobatannin, Gly. = Glycosides, Anthro. =Anthroquinone

Discussion

The present study developed an efficient protocol for *in vitro* germination, differentiation and phytochemical profiling of *A. ochracea*, representing the first detailed propagation study for this species in Bangladesh.

In vitro germination efficiency

Seed germination and seedling development of *A. ochracea* varied widely across the four basal media used, with PM medium performing best when supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. This combination yielded the fastest protocorm formation (13.03 weeks) and earliest leaf and root primordia development, leading to the highest overall seedling growth. The strong response in PM aligns with earlier orchid studies showing that PM enriched with cytokinin or organic additives supports rapid and high percentage germination in species such as *Cymbidium giganteum* [24] and *Dendrobium fimbriatum* [25]. Similar outcomes reported in *Cymbidium*, *Dendrobium*, *Arundina*, *Calanthe* and *Robiquetia* species [26-30] further confirm that PM, combined with balanced BAP-NAA, provides an optimal environment for orchid seedling morphogenesis.

Differentiation and growth responses

BAP in combination with NAA enhanced shoot multiplication, consistent with reports for *Dendrobium nobile* and *D. aphyllum* where meta-topolin and BAP stimulated rapid shoot proliferation [7, 31]. Auxin mediated rooting on IBA supplemented medium aligns with findings from *Paphiopedilum*, *Oncidium* and *Aerides* species [32-33]. Developmental progression from protocorm to leaf bearing seedling matched classical orchid morphogenesis described by Arditti and Ernst [6].

Hardening and survivability

The 82% survival rate demonstrates stability and adaptability of the regenerated plantlets. Coir-charcoal-brick substrates provide enhanced aeration and moisture, which have been widely recommended for acclimatizing epiphytic orchids [34-35].

Phytochemical significance

The qualitative analyses confirmed substantial accumulation of phenolics and flavonoids. Higher metabolite concentration in *in vitro* plants may be attributable to controlled nutrient and light conditions stimulating secondary metabolism [9, 36]. Enhanced metabolite production suggests that tissue culture derived plants may serve as a sustainable alternative source of medicinal compounds.

Conservation and application

Given the declining natural populations of *A. ochracea*, this protocol offers a robust framework for *ex situ* conservation, commercial propagation, pharmaceutical screening and future genetic improvement studies. By integrating *in vitro* germination with phytochemical enhancement, it underscores the species' dual significance in both conservation and biotechnological applications.

Conclusion

A complete *in vitro* propagation system for *A. ochracea* was established, including seed germination, protocorm differentiation and hardening. Phytochemical profiling confirmed the presence of biologically active metabolites at appreciable levels. The protocol is efficient, reproducible and suitable for conservation, research and pharmaceutical applications. Future work should include antioxidant activity assays, molecular authentication and optimization of metabolite production through elicitation.

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Conflicts of interest

The authors declare that there are no conflicts of interest associated with this publication.

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