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In vitro seed germination and phytochemical profiling of four epiphytic orchids of Chittagong Hill Tracts, Bangladesh

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Abstract
The Orchidaceae family represents a critical component of global biodiversity, yet many indigenous species in the Chittagong Hill Tracts (CHT) of Bangladesh face severe threats due to habitat destruction and over exploitation. This study investigates the *in vitro* seed germination efficiency, protocorm-like body (PLB) development, and comprehensive phytochemical profiling of four highly valued epiphytic orchids: *Aerides multiflora*, *Acampe rigida*, *Coelogyne flaccida* and *Dendrobium aggregatum*. A comparative analysis of seeds germination was conducted across four basal media (MS, KC, PM and MVW) supplemented with 2% or 3% (w/v) sucrose. The maximum seeds germination rate of 83.34% was recorded on the MS medium supplemented with 3% sucrose for *D. aggregatum*, outperforming all other media formulations. Subsequent PLBs development exhibited distinct species-specific morphological and pigmentation variations ranging from greenish to yellowish-white. Furthermore, extracts from naturally grown plant parts (leaf, stem/pseudobulb and root) and *in vitro* derived parts (callus, shoot buds, SPSs and plantlets) were subjected to rigorous qualitative screening for 15 secondary metabolites, including five alkaloids and ten additional classes (flavonoids, steroids, glycosides, anthraquinones, quinine, coumarins etc.). The profiling confirmed that *in vitro* generated plantlets successfully retain critical secondary metabolites (indicated by +, ++ and +++ colorimetric responses) comparable to their natural counterparts. These findings establish a highly efficient, reproducible protocol for the mass propagation and commercial phytochemical extraction of these epiphytic medicinal species, ensuring their *ex situ* conservation and sustainable pharmacological utilization.

Keywords: Epiphytic orchids, CHT, *In vitro* seeds germination, phytochemical profiling, PLBs, SPSs

Introduction

The Orchidaceae family, comprising over 35,000 species distributed across 800-850 genera, represents the largest and most diverse family of flowering plants on Earth (Arditti 1992) [2]. The beauty and mystique of orchids have long captivated both hobbyists and scientists. While orchids emerged during the Cretaceous period approximately 120 million years ago, their documented aesthetic and medicinal utilization dates back 3,000-4,000 years in China and the Vedic period (2000 BC to 600 BC) in India (Okhale *et al.* 2014) [14]. Today, orchids are among the most ecologically and evolutionarily significant plants, successfully colonizing almost all terrestrial ecosystems. Over 70% of tropical orchids exhibit an epiphytic growth habit, utilizing specialized aerial roots covered with spongy velamen tissues to absorb atmospheric moisture (Singh and Duggal 2009) [15].

Bangladesh, situated in a highly diverse climatic zone, hosts a rich variety of orchid species, particularly in the forests of the Chittagong Hill Tracts (CHT), Sylhet, and the Garo Hills (Huda *et al.* 2006) [10]. Despite their abundance, species such as *Aerides multiflora* Roxb., *Acampe rigida* (Buch. -Ham. ex Sm.), *Coelogyne flaccida* Lindl. and *Dendrobium aggregatum* Roxb. are facing severe threats. These four indigenous epiphytic orchids hold profound medicinal value. *A. multiflora* is traditionally utilized for wound healing and antibacterial treatments (Singh and Duggal 2009) [15]. *A. rigida* is applied to relax muscles, promote blood circulation and treat traumatic fractures (Huda and Kasem 2020) [9]. *C. flaccida* pseudobulb paste is historically used to cure headaches and fever (Subedi *et al.* 2013) [16]. *D. aggregatum* serves as a source of powerful analgesic, antipyretic and anti-inflammatory substances (Hossain 2013) [8].

Despite their immense pharmacological and commercial potential, the natural propagation of these orchids is severely restricted. Orchid seeds are microscopic, lack endosperm and possess a weak potential for germination. In the wild, they require a symbiotic relationship with specific mycorrhizal fungi to supply necessary nutrients for undifferentiated embryos to germinate (Goh 1982) [7]. Consequently, natural propagation is highly inefficient, necessitating the intervention of *in vitro* plant tissue culture techniques. *In vitro* propagation allows for the mass multiplication of rare and vulnerable orchids, bypassing the symbiotic requirement by providing artificial basal media enriched with essential macro/micronutrients and carbon sources (Arditti and Ernst 1993, Naing *et al.* 2011) [3, 13].

Beyond conservation, the phytochemical profile of these orchids is of immense scientific interest. Plants act as natural bio-factories, synthesizing secondary metabolites such as alkaloids, flavonoids, steroids and glycosides, which serve as defense mechanisms and offer significant therapeutic potential (Fransworth and Morris 1976, Velioglu *et al.* 1998) [6, 18]. Although natural harvesting of these compounds drives orchids toward extinction, *in vitro* tissue culture offers a sustainable alternative for harvesting bioactive components.

Therefore, the primary objectives of the current study are to evaluate the *in vitro* seed germination efficiency of *A. multiflora*, *A. rigida*, *C. flaccida* and *D. aggregatum* across different basal culture media and sucrose concentrations;

conduct a comprehensive qualitative screening of secondary metabolites to compare the phytochemical retention between naturally grown plant parts and *in vitro* derived tissues.

Materials and Methods

1. Plant Material Collection and Source of Explants

Four indigenous medicinal epiphytic orchid species: *Aerides multiflora* Roxb., *Acampe rigida* (Buch. -Ham. ex Sm.) P.F. Hunt., *Coelogyne flaccida* Lindl. and *Dendrobium aggregatum* Roxb. were selected for this study. Mature, undehisced capsules and plant parts of these species were collected from the hilly regions of the Chittagong Hill Tracts (CHT), Bangladesh. Taxonomic identification was confirmed based on the morphological features outlined in the Encyclopedia of Flora and Fauna of Bangladesh. Leaf, nodal and pseudobulb segments of *in vitro* grown seedlings were subsequently utilized as explants for propagation, with *in vitro* developed PLBs used for further multiplication.

2. Surface Sterilization Protocols

Collected capsules were initially washed under continuous running tap water to eliminate adhering debris. They were subsequently submerged in a mild detergent containing 2-4 drops of Tween 20 for 15 min and rinsed repeatedly. Aseptic sterilization was conducted inside a laminar airflow cabinet. The capsules were treated with a 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10 minutes, followed by a 70% (v/v) ethanol rinse for 30 seconds. Finally, the capsules were washed 3-4 times with sterile double-distilled water and dehydrated on sterile double layer Whatman filter paper to absorb surface moisture.

3. Basal Media Preparation

Four basal culture media were evaluated: MS (Murashige and Skoog 1962) [12], PM (Phytamax - Arditti 1977) [1], MVW (Modified Vacin and Went 1949) [17] and KC (Knudson 1946) [11].

- **MS Medium:** Prepared using concentrated stock solutions encompassing macronutrients, micronutrients, vitamins and inositol. It was supplemented with 3% (w/v) sucrose. The pH was adjusted to 5.8 using 1N NaOH or 1N HCl prior to the addition of 0.8% (w/v) agar.
- **PM, MVW and KC Media:** Prepared utilizing their respective stock solutions, supplemented with 2% (w/v) sucrose. For PM medium, 2.0 g/L of peptone was added. The pH for these three media was adjusted to 5.4.

All media were heated to dissolve the agar, dispensed into culture vessels and sterilized by autoclaving at 1.9 kg/cm² pressure at 121°C for 20 minutes.

4. Germination and Culture Conditions

Surface-sterilized capsules were longitudinally dissected using a sterile surgical blade. The powdery seeds were aseptically scooped and evenly distributed onto the surface of the solidified culture media. The culture vessels were incubated in a highly controlled growth room maintained at 25 ± 2°C with a relative humidity of 50-60%. A photoperiod of 14 hours of continuous light (4000-5000 lux from cool white fluorescent tubes) and 10 hours of darkness was maintained.

5. Phytochemical Extract Preparation

Extracts were prepared from naturally grown plant parts (leaf, stem, and root) and *in vitro* developed tissues (callus, SPSs, shoot buds and plantlets). For *C. flaccida*, pseudobulbs were utilized instead of stems due to its specific morphological habit.

- **Extraction:** 5g of fresh, finely chopped plant material was mixed with 10 mL of 2% HCl and heated in a water bath at 60ºC for one hour. Following cooling, the extract was filtered through Whatman No.1 filter paper.

- **Qualitative Profiling:** The extracts were subjected to spot tests to detect five specific alkaloids using Cromwell's (1955) [5] reagents: Dragendorff's, Hager's, Mayer's, Tannic acid and Wagner's reagent. Additionally, standard biochemical protocols were employed to screen for Phlobatannin, Flavonoids, Saponins, Tannins, Terpenoids, Steroids, Glycosides, Anthraquinone, Quinine and Coumarin. The relative abundance of precipitates/ colorimetric changes was recorded as '+' (slight), '++' (moderate), '+++' (substantial), or '–' (absent).

6. Acclimatization

Robust, rooted *in vitro* plantlets were transferred to *ex vitro* conditions following a strict hardening protocol. The culture vessels were initially kept open for 24 hours in the culture room. Plantlets were then carefully extracted, and the roots were washed under running water to remove residual agar. They were transplanted into small earthen pots containing a specialized potting mixture: small bricks, sawdust, coconut husk, activated charcoal, and hardwood coal in a 1:1:1:1:1 ratio. The mixture was pre-treated with 0.1% Agrosan fungicide. Pots were maintained in a greenhouse at 25-30°C and 60-70% RH, with regular watering over 2-3 months until full establishment.

7. Experimental Design and Statistical Analysis

All experiments were designed following a Completely Randomized Design (CRD) and replicated three times. The parameters included the percentage of seed germination, PLBs formation rate and phytochemical abundance. Statistical significance was evaluated using one-way ANOVA, with mean separations performed using Duncan's Multiple Range Test (DMRT). Data were represented as means ± standard error.

Results

1. *In vitro* Seed Germination Efficiency and PLBs Development

The seed germination efficiency across the four selected epiphytic orchids was heavily influenced by the type of basal nutrient medium and the concentration of sucrose (Table 1). The MS medium consistently outperformed KC, PM and MVW media across all species tested except *A. rigida* in PM medium. Specifically, PM medium supplemented with 2% (w/v) sucrose yielded the highest germination rates, of 75% for *A. rigida* (Fig. 1). Wherwas, optimal trends were observed for *A. multiflora* (91.67%, Fig. 2), *C. flaccida* (91.67%, Fig. 3) and *D. aggregatum* (100%, Fig. 4) on the MS medium with 3% sucrose.

Conversely, media utilizing 2% (w/v) sucrose exhibited moderate to low germination efficiencies except *A. rigida*. The KC medium showed the lowest overall germination

success, yielding a minimal germination for *A. multiflora* (58.34%), *A. rigida* (33.34%), *C. flaccida* (50.00%) and *D. aggregatum* (58.34%). The initiation time for germination varied depending on the species and medium, with the earliest swelling and initial greening of embryos occurring between 25 to 35 days on MS medium, while seeds on KC and MVW media delayed up to 45 days.

Following germination, the undifferentiated embryos developed into protocorm-like bodies (PLBs). Morphological and pigmentation changes during this phase were distinct among the species. *D. aggregatum* PLBs transitioned from a pale white to a vibrant greenish-yellow. *A. multiflora* developed prominent greenish PLBs, whereas *A. rigida* displayed yellowish-white pigmentation during early protocorm development. *C. flaccida* formed robust yellowish-green PLBs before differentiating into early plantlets. The PLBs formed on MS medium were visibly larger, denser and possessed superior organogenic capacity compared to those on PM or MVW media except *A. rigida* in PM medium.

2. Phytochemical Profiling

The qualitative presence of secondary metabolites was systematically compared between naturally grown plant parts and *in vitro* derived parts. Results highlighted both species-specific variations and a strong retention of bioactive compounds in tissue cultured samples.

Aerides multiflora (Tables 2 & 3)

Analysis of natural *A. multiflora* parts (leaf, stem, root) revealed the moderate (++) presence of alkaloids *via* Wagner's and Dragendorff's reagents, primarily localized in the leaves. Saponins, flavonoids and glycosides were abundant (+++) in the stem. In contrast, the *in vitro* parts (callus, SPSs, shoot buds, plantlets) successfully mirrored this profile. *In vitro* plantlets exhibited a strong (+++) presence of flavonoids and tannins, proving that tissue culture retains the medicinal efficacy of the natural plant.

Acampe rigida (Tables 4 & 5)

Naturally grown *A. rigida* leaves demonstrated a substantial (+++) concentration of steroids, terpenoids and coumarins, justifying its traditional use for joint pain and blood circulation. Alkaloids tested positive (+) primarily with Mayer's reagent in root extracts. The methanolic extracts of *in vitro* SPSs and shoot buds showed a parallel synthesis of terpenoids (++) and steroids (++). Notably, anthraquinones were absent (–) in both natural and *in vitro* extracts of this species.

Coelogyne flaccida (Tables 6 & 7)

For *C. flaccida*, natural pseudobulbs were utilized instead of stems. The natural pseudobulb extracts were highly concentrated (+++) with alkaloids (confirmed strongly by Hager's and Tannic acid reagents), glycosides and quinine. The *in vitro* developed callus and differentiated plantlets synthesized equivalent levels of glycosides (+++) and moderate levels (++) of alkaloids. The retention of these specific metabolites in *in vitro* SPSs highlights the viability of commercial extraction from tissue-cultured clones without harvesting wild populations.

Dendrobium aggregatum (Tables 8 & 9)

D. aggregatum exhibited the most diverse phytochemical profile. Natural stems and leaves showed substantial (+++) responses for flavonoids, saponins, phlobatannins and alkaloids (positive across all five reagents). The *in vitro* plantlets and shoot buds demonstrated a highly active secondary metabolism, retaining the maximum (+++) presence of flavonoids and saponins and moderate (++) alkaloid content. The presence of quinine and coumarins was also noted (+) in *in vitro* plantlets, matching the biochemical signature of the wild harvested samples.

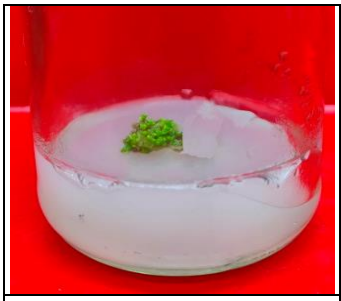

Fig 1: Seeds germination of *A. rigida* on PM medium

Fig 2: Seeds germination of *A. multiflora* on MS medium

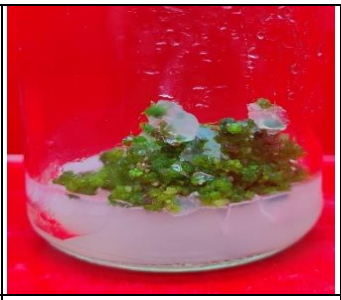

Fig 3: Seeds germination of *C. flaccida* on MS medium

Fig 4: Seeds germination of *D. aggregatum* on MS medium

Table 1: *In vitro* seeds germination of four epiphytic orchids

Species name	Nutrient medium	Carbohydrate conc.	Number of culture vessels used	Number of culture vessels in which seeds germinated		PLBs Colour	Remarks
				No.	%		
Aerides multiflora	KC	2% (w/v) sucrose	12	07	58.34	Yellowish green	+
	MS	3% (w/v) sucrose	12	11	91.67	Greenish	+++
	PM	2% (w/v) sucrose	12	10	83.34	Greenish yellow	+++
	MVW	2% (w/v) sucrose	12	08	66.67	Yellowish white	++
Acampe rigida	KC	2% (w/v) sucrose	12	04	33.34	Yellowish white	+
	MS	3% (w/v) sucrose	12	08	66.67	Greenish yellow	++
	PM	2% (w/v) sucrose	12	09	75.00	Greenish	++

	MVW	2% (w/v) sucrose	12	05	41.67	Yellowish green	+
Coelogyne flaccida	KC	2% (w/v) sucrose	12	06	50.00	Yellowish white	+
	MS	3% (w/v) sucrose	12	11	91.67	Greenish	+++
	PM	2% (w/v) sucrose	12	10	83.34	Greenish yellow	+++
	MVW	2% (w/v) sucrose	12	08	66.67	Yellowish green	++
Dendrobium aggregatum	KC	2% (w/v) sucrose	12	07	58.34	Yellowish white	++
	MS	3% (w/v) sucrose	12	12	100.00	Greenish	+++
	PM	2% (w/v) sucrose	12	11	91.67	Greenish yellow	+++
	MVW	2% (w/v) sucrose	12	09	75.00	Yellowish green	++

Values represent mean ± SE of each experiment consist of 12 replicates.

Table 2: Qualitative Phytochemical profiling (alkaloids) of naturally grown Leaf, Stem, Root and *in vitro* developed Callus, SPSs & Shoot bud of *Aerides multiflora*

Plant type	Plant Parts Used	Qualitative Estimation of Alkaloids				
		D	H	M	T	W
Natural	Leaf	++	+	+	+++	++
	Stem	+++	+++	+	+++	++
	Root	+++	+++	++	+++	++
In vitro	Callus	++	+	+	+++	++
	SPSs	+++	+++	++	+++	+++
	Shoot buds	+++	+++	++	+++	+++

Notes: Name of reagents- D- Dragendroff's reagent, H- Hager's reagent, M- Mayer's reagent, T- Tannic acid reagent and W- Wagner's reagent. Here, "+++" means highest result, "++" means medium result, "+" means lowest result.

Table 3: Qualitative test of ten other phytochemicals of naturally grown Leaf, Stem, Root and *in vitro* developed Plantlets of *Aerides multiflora*

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

Notes: Phl. = Phlobatannins, Flv. = Flavonoids, Sap. =Saponins, Tan. = Tanins, Ter. =Terpinoids, Str. =Steroids, Gly. = Glycosides, Ant. =Anthroquinone, Qui. =Quinine, Cou. = Coumarin. Here, "+++" means highest response, "++" means medium response, "+" means lowest response and "-" means absent.

Table 4: Qualitative Phytochemical profiling (alkaloids) of naturally grown Leaf, Stem, Root and *in vitro* developed Callus, SPSs & Shoot bud of *Acampe rigida*

Plant type	Plant parts used	Qualitative estimation of alkaloids				
		D	H	M	T	W
Natural	Leaf	++	+++	+++	+++	++
	Stem	+++	+++	++	+++	++
	Root	++	++	+++	+++	++
In vitro	Callus	++	+++	+++	++	+++
	SPSs	+++	++	+++	+++	++
	Shoot buds	++	++	+++	+++	+++

Notes: Name of reagents- D- Dragendroff's reagent, H- Hager's reagent, M- Mayer's reagent, T- Tannic acid reagent and W- Wagner's reagent. Here, "+++" means highest result, "++" means medium result, "+" means lowest result.

Table 5: Qualitative test of ten other phytochemicals of naturally grown Leaf, Stem, Root and *in vitro* developed Plantlets of *Acampe rigida*

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

Notes: Phl. = Phlobatannins, Flv. = Flavonoids, Sap. =Saponins, Tan. = Tanins, Ter. =Terpinoids, Str. =Steroids, Gly. = Glycosides, Ant. =Anthroquinone, Qui. =Quinine, Cou. = Coumarin. Here, "+++" means highest response, "++" means medium response, "+" means lowest response and "-" means absent.

Table 6: Qualitative Phytochemical profiling (alkaloids) of naturally grown Leaf, Pseudo-bulb, Root and *in vitro* developed Callus, SPSs & Shoot bud of *Coelogyne flaccida*

Plant type	Plant parts used	Qualitative estimation of alkaloids				
		D	H	M	T	W
Natural	Leaf	+	+++	++	++	+++
	Pseudo-bulb	++	++	++	+++	+++
	Root	+++	+++	+++	++	+++
In vitro	Callus	++	+	+	+	++
	SPSs	+	+	++	++	+
	Shoot buds	+++	++	+++	+++	+++

Notes: Name of reagents- D- Dragendroff's reagent, H- Hager's reagent, M- Mayer's reagent, T- Tannic acid reagent and W- Wagner's reagent. Here, "+++" means highest result, "++" means medium result, "+" means lowest result.

Table 7: Qualitative test of ten other phytochemicals of naturally grown Leaf, Pseudo-bulb, Root and *in vitro* developed Plantlets of *Coelogyne flaccida*

Plant type	Plant parts used	Secondary metabolites (% of coloration)									
		Phl.	Flv.	Sap.	Tan.	Ter.	Str.	Gly.	Ant.	Qui.	Cou.
Natural	Leaf	+++	+	+	+	++	+	-	+	-	++
	Pseudo-bulb	+	+++	-	++	+	+	+++	++	-	+
	Root	++	++	-	+	++	-	++	-	-	+
In vitro	Plantlets	+++	-	-	++	++	++	+	-	-	+

Notes: Phl. = Phlobatannins, Flv. = Flavonoids, Sap. =Saponins, Tan. = Tanins, Ter. =Terpinoids, Str. =Steroids, Gly. = Glycosides, Ant. =Anthroquinone, Qui. =Quinine, Cou. = Coumarin. Here, "+++" means highest response, "++" means medium response, "+" means lowest response and "-" means absent.

Table 8: Qualitative Phytochemical profiling (alkaloids) of naturally grown Leaf, Stem, Root and *in vitro* developed Callus, SPSs & Shoot bud of *Dendrobium aggregatum*

Plant type	Plant parts used	Qualitative estimation of alkaloids				
		D	H	M	T	W
Natural	Leaf	+	+	+	++	+++
	Stem	+++	++	++	+++	+++
	Root	+++	++	+	++	+++
In vitro	Callus	+++	+	+	++	++
	SPSs	+++	++	+	+++	++
	Shoot buds	++	++	++	+++	+++

Notes: Name of reagents- D- Dragendroff's reagent, H- Hager's reagent, M- Mayer's reagent, T- Tannic acid reagent and W- Wagner's reagent. Here, "+++" means highest result, "++" means medium result, "+" means lowest result.

Table 9: Qualitative test of ten other phytochemicals of naturally grown Leaf, Stem, Root and *in vitro* developed Plantlets of *Dendrobium aggregatum*

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

Notes: Phl. = Phlobatannins, Flv. = Flavonoids, Sap. =Saponins, Tan. = Tanins, Ter. =Terpinoids, Str. =Steroids, Gly. = Glycosides, Ant. =Anthroquinone, Qui. =Quinine, Cou. = Coumarin. Here, "+++" means highest response, "++" means medium response, "+" means lowest response and "-" means absent.

Discussion

The successful conservation and commercial utilization of endangered epiphytic orchids rely heavily on deciphering optimal *in vitro* germination requirements and validating their pharmacological retention. This study established that seeds germination and subsequent morphogenetic responses are intricately tied to the basal medium's biochemical composition.

The pronounced superiority of the MS medium, which yielded a maximum seed germination rate for *A. multiflora* (91.67%), *C. flaccida* (91.67%) and *D. aggregatum* (100%) except *A. rigida* (75%) in PM medium at a 3% sucrose concentration, can be attributed to its high concentration of macronutrients, particularly its total nitrogen content in the form of ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3). Orchid seeds, lacking an endosperm, require an external, easily metabolizable carbon source. The 3% sucrose concentration in the MS medium provided the optimal osmotic potential and energy supply required for rapid cell division and protocorm differentiation. This aligns with findings by Chugh *et al.* (2009) [4], who reported that nitrogen-rich media accelerate the organogenesis of epiphytic orchid PLBs. Conversely, the lower germination rates observed in KC and MVW media are likely due to

their lower ionic strength and reduced macronutrient profiles, which fail to meet the intense metabolic demands of early embryo swelling.

The morphological variations and pigmentation shifts observed during PLBs development (ranging from greenish-yellow in *D. aggregatum* to yellowish-white in *A. rigida*) reflect species-specific chlorophyll biosynthesis rates in response to the *in vitro* light regime and media nitrogen levels. The addition of organic supplements like peptone in the PM medium provided a slight boost to PLBs robustness, but MS remained the superior basal formulation for structural differentiation into early plantlets.

A critical breakthrough in this study is the validation of the phytochemical integrity of *in vitro* propagated orchids. The qualitative profiling unequivocally demonstrated that *in vitro* generated tissues including callus, SPSs and fully differentiated plantlets are highly capable of synthesizing complex secondary metabolites independently of their natural ecological niche. For instance, the significant (+++) retention of glycosides and alkaloids in the *in vitro* pseudobulbs of *C. flaccida* mirrors the chemical profile of wild specimens used in traditional medicine for headaches and fevers (Subedi *et al.* 2013) [16]. Similarly, the high flavonoid and saponin content recorded in *D. aggregatum in vitro* plantlets confirms that tissue culture does not compromise the plant's anti-inflammatory and analgesic properties.

These findings suggest that environmental stressors and symbiotic fungal interactions, while crucial in nature (Fransworth and Morris 1976) [6], are not strictly requisite for the biosynthesis of therapeutic compounds like terpenoids, steroids and coumarins in these species, provided the *in vitro* nutrient matrix is optimized. A minor limitation of the study is the qualitative nature of the phytochemical screening; however, the strong colorimetric indicators (+ to +++) provide a foundational baseline for future High-Performance Liquid Chromatography (HPLC) quantification. The successful ex *vitro* acclimatization of these robust plantlets using a custom aggregate mixture of bricks, charcoal and coconut husk further proves that this protocol is ready for commercial scaling.

Conclusion

This study successfully establishes a highly efficient and reproducible protocol for the *in vitro* mass propagation and phytochemical validation of four endangered medicinal orchids from the Chittagong Hill Tracts: *Aerides multiflora*, *Acampe rigida*, *Coelogyne flaccida* and *Dendrobium aggregatum*. The formulation of MS medium with 3% sucrose proved exceptionally effective, achieving seeds germination rate in all of the selected species except *A. rigida*. Furthermore, comprehensive phytochemical screening confirmed that *in vitro* propagated plantlets successfully retain vital secondary metabolites including alkaloids, flavonoids and glycosides at concentrations comparable to their wild counterparts. This breakthrough dual purpose protocol provides a sustainable pathway for the commercial pharmaceutical exploitation of these species while simultaneously mitigating wild harvesting pressures, thereby ensuring their long-term *ex situ* conservation in Bangladesh.

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