



***In vitro* seed germination and phytochemical profiling of *Dendrobium farmeri* Paxton, an epiphytic orchid of Bangladesh**

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Abstract

Dendrobium farmeri Paxton is an epiphytic, medicinally significant, and endangered orchid species native to the tropical forests of Bangladesh. The natural propagation of this species is severely constrained due to its minute, endosperm-less seeds that require specific mycorrhizal fungal associations for germination. Furthermore, indiscriminate wild harvesting for its therapeutic secondary metabolites has critically depleted its natural populations. This study was undertaken to establish an efficient *in vitro* asymbiotic seed germination protocol for *D. farmeri* and to conduct a comparative qualitative phytochemical screening between naturally grown plant parts and *in vitro* derived plantlets. Seed germination efficiency were evaluated across four basal nutrient media: Murashige and Skoog (MS), Knudson C (KC), Phytamax (PM) and Modified Vacin and Went (MVW). The highest seed germination rate of 83.34% was achieved on MS medium produced healthy greenish PLBs. Conversely, KC medium exhibited the lowest germination efficiency at 41.67%. Subsequent phytochemical investigations the presence of alkaloids and eleven other secondary metabolites. Spot tests revealed that naturally grown pseudobulbs and leaves possessed robust concentrations of alkaloids, terpenoids, tannins and phenols. Crucially, the *in vitro* derived plantlets successfully retained substantial quantities of key therapeutic metabolites, notably alkaloids, terpenoids and quinine, while lacking anthraquinones mirroring the chemical profile of wild counterparts. These findings validate *in vitro* propagation not only as an essential conservation strategy for endangered epiphytic orchids but also as a highly viable, sustainable alternative for the continuous commercial production of valuable phytochemicals, reducing ecological pressure on wild *D. farmeri* populations.

Keywords: *Dendrobium farmeri*, *In vitro* seed germination, Conservation, Phytochemical profiling, Secondary metabolites

Introduction

The Orchidaceae family represents one of the most evolutionarily advanced and aesthetically diverse botanical groups on Earth, comprising over 779 genera and an estimated 25,000 to 35,000 species worldwide (Mabberley 2008, Chen *et al.* 2009, Bektas *et al.* 2013) [3, 6, 14]. While cosmopolitan in distribution, orchids are predominantly concentrated in the tropical and subtropical belts of Asia, with Bangladesh hosting approximately 187 documented species primarily distributed across the evergreen and semi-evergreen rain forests of the Chittagong Hill Tracts, Cox's Bazar and Sylhet (Rahman *et al.* 2017) [18]. Among these, the genus *Dendrobium* is highly prized for both its profound horticultural appeal and extensive ethno-medicinal utility.

Dendrobium farmeri Paxton is a sympodial, epiphytic orchid characterized by pendulous, clavate to fusiform pseudobulbs with deeply sulcate internodes, yielding striking racemes of pinkish-mauve and yellow blooms. Beyond its ornamental value, *D. farmeri* possesses immense therapeutic potential. Historical and ethno-pharmacological literature indicates that *Dendrobium* species (traditionally referred to as *shi-hu* in Chinese medicine) have been utilized for millennia to treat a myriad of ailments, possessing anti-inflammatory, antimicrobial, and antioxidant properties driven by their complex biochemical profiles (Hossain 2011, De 2022) [7, 10]. However, rampant habitat fragmentation, illegal cross-border plant trafficking and unsustainable wild harvesting have critically endangered *D. farmeri* within its natural geographical range in Bangladesh (Phelps and Webb 2015, Rashid *et al.* 2017) [17, 18].

The natural regeneration of *D. farmeri* is biologically impeded by the unique morphology of orchid seeds. Orchid capsules release hundreds of thousands of microscopic, dust-like seeds that entirely lack an endosperm (Roberts and Dixon 2008) [21]. Consequently, seeds require a highly specific symbiotic relationship with mycorrhizal fungi to metabolize complex environmental carbohydrates into accessible simple sugars for germination (Rasmussen 1995) [20]. In the absence of these precise ecological conditions, wild germination rates are exceptionally low. To circumvent these biological bottlenecks and mitigate the threat of extinction, *in vitro* asymbiotic seed germination and tissue culture techniques have emerged as indispensable tools for large-scale micropropagation and germplasm conservation (Arditti and Ernst 1993) [2]. Previous studies have demonstrated that variations in basal media formulations particularly the concentrations of macronutrients, micronutrients and carbohydrate sources profoundly dictate the morphogenetic differentiation of orchid seeds into protocorm-like bodies (PLBs) and subsequent plantlets (Chen and Chang 2000 [5], Nayak *et al.* 1997).

Furthermore, while *in vitro* propagation secures the botanical survival of the species, establishing its pharmacological viability requires rigorous phytochemical validation. Plants defend against biotic and abiotic stressors *via* the synthesis of secondary metabolites, such as alkaloids, flavonoids, terpenoids and saponins, which form the biochemical basis for their medicinal efficacy. Contemporary botanical research increasingly focuses on determining whether *in vitro* cultured tissues can effectively

synthesize and accumulate these bioactive compounds at levels comparable to wild-harvested specimens (Bhattacharyya *et al.* 2016^[4], De 2022). If *in vitro* plantlets of *D. farmeri* can be proven to biosynthesize equivalent therapeutic metabolites, tissue culture could sustainably supply the pharmaceutical industry while strictly preserving wild populations.

Therefore, the explicit objectives of this investigation were twofold: first, to systematically evaluate and standardize the *in vitro* seed germination efficiency and PLBs development of *D. farmeri* across four distinct basal culture media (MS, KC, PM and MVW) and second, to conduct a comprehensive qualitative phytochemical screening to compare the secondary metabolite profiles of naturally grown plant parts (leaves, roots and pseudobulbs) with those of *in vitro* derived plantlets.

Materials and Methods

1. Plant Material Collection and Taxonomic Identification

Mature, intact green capsules of *Dendrobium farmeri* Paxton, alongside samples of naturally grown leaves, roots and pseudobulbs were collected from the wild epiphytic habitats of Manikpur, Chakaria, Cox's Bazar, Bangladesh. The taxonomic identity of the species was formally authenticated based on morphological characteristics (coriaceous oval-lanceolate leaves, 4-angled sulcate stems) prior to the commencement of *in vitro* and phytochemical experiments.

2. Surface Sterilization Protocol

To prevent microbial contamination during *in vitro* inoculation, a stringent surface sterilization protocol was employed. The intact capsules were initially scrubbed under running tap water to remove superficial dust and debris, followed by 3-4 thorough washes with sterile distilled water. The capsules were then gently rubbed with savlon-soaked cotton and rinsed an additional 2-3 times with distilled water. Chemical surface sterilization was achieved by treating the capsules with a 0.2% (w/v) mercuric chloride (HgCl₂) solution for 5 minutes, followed by 2-3 washes with double sterile distilled water inside a laminar airflow cabinet. Final disinfection was executed by immersing the capsules in 70% ethanol for 1 minute, succeeded by a final sequence of 2-3 rinses in double sterile distilled water.

3. Basal Media Preparation for Seed Germination

Four distinct basal nutrient media were prepared to evaluate asymbiotic germination efficiency: Knudson C-KC (Knudson 1946)^[12], Murashige and Skoog-MS (Murashige and Skoog 1962), Phytamax-PM (Arditti 1977)^[1, 15], Modified Vacin and Went-MVW (Vacin and Went 1949)^[24]. 3% (w/v) sucrose supplementation used in MS medium while others three medium fortified with 2% (w/v) sucrose. Standard stock solutions of macronutrients, micronutrients, vitamins, and organic supplements were formulated for each medium.

Carbohydrate supplementation varied according to the specific medium protocol; MS medium was supplemented with 3% (w/v) sucrose, while PM, MVW and KC media were supplemented with 2% (w/v) sucrose. Additionally, 2.0 g/l of peptone was integrated into the PM medium. To solidify the media, 0.8% (w/v) agar (Fluka) was utilized for the KC, MS, PM and MVW media. Prior to the addition of

agar, the pH of the media was strictly adjusted using a digital pH meter (TOA HM-30V) and 1N NaOH or 1N HCl: MS was adjusted to pH 5.8, PM and MVW to pH 5.4, and KC to pH 5.0. Medium (30 ml for test tubes and 50 ml for conical flasks) were dispensed into culture vessels, tightly plugged with cotton, sealed with aluminum foil and autoclaved at a pressure of 1.9 Kg/cm² at 121°C for 20 minutes.

4. *In vitro* Seed Inoculation and Incubation

Aseptic longitudinal dissection of the sterilized capsules was performed using a sterile surgical blade on an aluminum slab within the laminar airflow cabinet. The microscopic, powdery seeds were meticulously scooped using sterile forceps and evenly broadcasted over the surface of the solidified germination media. The inoculated culture vessels were then transferred to a controlled culture room maintained at a temperature of 25±2°C. The photoperiod was regulated at 14 hours of continuous light and 10 hours of continuous dark phase. Data regarding germination initiation, protocorm-like bodies (PLBs) formation and morphological pigmentation were recorded sequentially. The experimental design was a Completely Randomized Design (CRD) featuring 12 replicates for each of the four media treatments.

5. Phytochemical Extract Preparation

Qualitative phytochemical screening was executed on both the wild-harvested plant parts (leaves, natural pseudobulbs and roots) and the *in vitro* derived plantlets (including cultured callus, shoot buds and SPSs for specific alkaloid tests). Methanolic crude extracts were prepared to screen for general secondary metabolites. Freshly collected and *in vitro* plant tissues were finely chopped, shade-dried, and ground into a crude powder. Approximately 0.5 g of the crude powder from each specific plant part was dissolved in 5 ml of high-purity methanol. The active biological compounds were extracted by evaporating the methanol under reduced pressure utilizing a Vacuum Rotary Evaporator, yielding the concentrated crude extracts utilized for spot testing.

6. Qualitative Phytochemical Profiling

Standardized colorimetric methodologies were employed to detect the qualitative presence and relative abundance (+, ++, +++) of twelve major phytochemical classifications.

- **Alkaloids:** A specialized rapid spot test was conducted where 5g of fresh, pasted plant material was mixed with 10 ml of 2% HCl and heated in a water bath at 60°C for 1 hour. Following filtration, two drops of the extract were reacted on a groove slide with specific alkaloid-detecting reagents: Dragendorff's reagent (forming water-red turbidity/precipitate), Hager's reagent (yellow precipitate), Mayer's reagent (white/cloudy precipitate), Wagner's reagent (brown flocculent precipitate) and Tannic acid reagent.
- **Phlobatannins:** The aqueous extract was boiled with 1% aqueous HCl; the deposition of a red precipitate indicated a positive presence (Edeoga *et al.* 2005)^[8].
- **Anthraquinones:** Treating 2 ml of extract with magnesium acetate solution; development of a pink or red coloration indicates anthraquinones (Sofowara 1993)^[22].

- **Flavonoids:** The crude powder was heated with ethyl acetate over a steam bath, filtered, and shaken with dilute ammonia solution. The development of a yellow coloration verified the presence of flavonoids. (Edeoga *et al.* 2005)^[8].
- **Saponins:** Chambering 2g crude powder with 20 ml distilled water in a hot water bath, followed by vigorous shaking of 10 ml filtrate with 5 ml water. Persistence of stable, thick froth indicates saponins (Kapoor *et al.* 1969)^[11].
- **Tannins:** Boiling 0.5g of powder in 10 ml distilled water, filtering, and adding a few drops of 0.1% ferric chloride (FeCl₃) reagent. Blue-black or green precipitate indicates tannins (Harborne 1973)^[9].
- **Terpenoids:** 5 ml of methanolic extract treated with 2 ml chloroform, followed by careful addition of 3 ml concentrated sulfuric acid (H₂SO₄) down the side. A reddish-brown ring interface indicates terpenoids (Kolawole *et al.* 2006)^[13].
- **Steroids:** Exposing 1 ml of methanolic extract to 10 ml chloroform and adding an equal volume of concentrated H₂SO₄ down the side. Red coloration in the upper phase and yellow-green fluorescence in the acid phase indicates steroids (Kolawole *et al.* 2006)^[13].
- **Glycosides:** Two ml of extract heated with 10 ml 50% HCl in a boiling water bath for 30 min, followed by the addition of 5 ml Fehling's solution and boiling for 5 min. Appearance of a brick-red precipitate indicates glycosides (Harborne 1973)^[9].
- **Quinine:** Adding 1 ml concentrated H₂SO₄ to 1 ml extract; development of a red color indicates quinine (Sofowara 1993)^[22].
- **Coumarins:** Mixing 1 ml extract with 1 ml 10% NaOH; development of a yellow color indicates coumarins (Sofowara 1993)^[22].
- **Phenols:** Ferric chloride testing of methanolic extract; development of a deep green or bluish-black coloration indicates phenols (Harborne 1973)^[9].

7. Statistical Analysis

The mean percentage of seed germination across the culture vessels was computed. Data points were expressed of the number of observations (N = 12 replicates per treatment).

Results

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

The asymbiotic germination of *D. farmeri* seeds successfully occurred across all four evaluated basal nutrient media; however, substantial variances in germination efficiency and protocorm morphogenesis were observed based on the specific media composition and carbohydrate concentrations (Table 1).

The maximum seed germination efficiency was recorded on the MS medium supplemented with 3% (w/v) sucrose, which achieved an 83.34% germination rate (Fig. 1).

Furthermore, the differentiating protocorm-like bodies (PLBs) cultured on the MS medium exhibited a highly viable, robust greenish pigmentation indicative of active photosynthetic capability. The Phytamax (PM) medium, containing 2% (w/v) sucrose, yielded the second highest germination rate at 66.67%, accompanied by the formation of greenish-yellow PLBs (Fig. 2). The Modified Vacin and Went (MVW) medium recorded a moderate germination frequency of 58.34%, yielding yellowish-green PLBs (Fig. 3). Conversely, the KC medium demonstrated the poorest germination response at just 41.67%, with the resulting PLBs displaying a pale, yellowish-white morphology (Fig. 4). Following initial differentiation, the numerous miniature shoot buds formed from the germinated PLBs on the superior media were successfully established for continuous *in vitro* growth.

2. Qualitative Phytochemical Profiling

A comprehensive comparative phytochemical screening was conducted to ascertain the synthesis and retention of medicinal secondary metabolites within the natural vegetative organs (leaves, pseudobulbs, roots) versus the *in vitro* developed tissues of *D. farmeri*.

2.1 Alkaloid Detection

The qualitative presence of alkaloids was evaluated utilizing five distinct biochemical reagents, confirming that both natural and *in vitro* tissues possess active alkaloidal constituents, albeit with varying intensities (Table 2).

Among the naturally grown tissues, the pseudobulbs exhibited the highest relative abundance of alkaloids, showing maximal responses (+++) to Dragendorff's, Wagner's, and Tannic acid reagents, and moderate responses (++) to Hager's and Mayer's. Natural leaves showed robust concentrations primarily when tested with Dragendorff's and Wagner's reagents (+++), while natural roots generally exhibited low alkaloid detection (+), except for a moderate response to Wagner's reagent (++) . Crucially, the *in vitro* developed tissues demonstrated remarkable alkaloid retention. The *in vitro* derived shoot buds and SPSs (secondary protocorm-like bodies/shoots) displayed maximum (+++) responses across multiple reagents (Wagner's, Tannic Acid and Dragendorff's), verifying that the *in vitro* culture protocol successfully sustains the metabolic pathways required for alkaloid biosynthesis.

2.2. Screening of Major Secondary Metabolites

The methanolic crude extracts were further subjected to spot assays for eleven primary pharmacological metabolite groups (Table 3).

The screening unequivocally demonstrated the omnipresence of high terpenoid concentrations (+++) across all biological samples, both wild and *in vitro*. Similarly, anthraquinones were consistently absent (-) across the entirety of the *D. farmeri* samples analyzed.

Naturally grown leaves were uniquely characterized by the highest relative abundance (+++) of flavonoids, tannins, terpenoids, quinine and phenols, while possessing the lowest baseline concentration (+) of coumarin. The natural pseudobulbs registered maximum concentrations (+++) of phlobatannins, tannins, terpenoids and steroids, exhibiting a broadly balanced metabolic profile. Natural roots showed the highest presence of phlobatannins, terpenoids, steroids,

quinine and phenol, but notably lacked coumarins completely (-).

Significantly, the whole *in vitro* derived plantlets yielded an exceptionally strong phytochemical profile. They expressed maximum (+++) levels of quinine and terpenoids, directly rivaling the wild-grown roots and leaves. Furthermore, the *in vitro* plantlets successfully synthesized moderate (++)

quantities of phlobatannins, saponins, tannins, steroids, glycosides, coumarins and phenols. Flavonoids were detected at lower (+) thresholds within the tissue-cultured samples. Overall, the qualitative bio-assay provides empirical evidence that *in vitro* propagation does not critically diminish the fundamental secondary metabolite cascade of *D. farmeri*.

Table 1: *In vitro* germination of seeds of *D. farmeri* Paxton

Nutrient medium	Carbohydrate conc.	Number of culture vessels used	Number of culture vessels in which seeds germinated		PLBs Colour	Remarks
			No.	%		
KC	2% (w/v) sucrose	12	05	41.67	Yellowish white	+
MS	3% (w/v) sucrose	12	10	83.34	Greenish	++
PM	2% (w/v) sucrose	12	08	66.67	Greenish yellow	++
MVW	2% (w/v) sucrose	12	07	58.34	Yellowish green	+

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

Table 2: Qualitative test for alkaloids of natural and *in vitro* grown plant parts of *D. farmeri*

Plant type	Plant parts used	Qualitative estimation of alkaloids by different reagents				
		D	H	M	W	T
Natural	Leaf	+++	+	+	+++	++
	Pseudobulb	+++	++	++	+++	+++
	Root	+	+	+	++	+
<i>In vitro</i>	Callus	+++	+	+	++	++
	Shoot bud	++	++	++	+++	+++
	SPSs	+++	++	+	++	+++

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 response, "++" means medium response, "+" means lowest response.

Table 3: Qualitative test of eleven secondary metabolites of naturally grown and *in vitro* plantlets of *D. farmeri*.

Plant parts used	Secondary metabolites (% of coloration)										
	Phl.	Flv.	Sap.	Tan.	Ter.	Str.	Gly.	Ant.	Qui.	Cou.	Phe.
Leaf	++	+++	++	+++	+++	++	++	-	+++	+	+++
Pseudobulb	+++	+	++	+++	+++	+++	++	-	++	++	++
Root	+++	+	++	++	+++	+++	++	-	+++	-	+++
<i>In vitro</i> plantlets	++	+	++	++	+++	++	++	-	+++	++	++

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



Fig 1: *In vitro* seed germination of *Dendrobium farmeri* on MS medium.

Fig 2: *In vitro* seed germination of *D. farmeri* on PM medium.

Fig 3: *In vitro* seed germination of *D. farmeri* on MVW medium.

Fig 4: *In vitro* seed germination of *D. farmeri* on KC medium.

Discussion

The reliance of wild orchid populations on highly specialized symbiotic mycorrhizal associations, combined with the absence of endosperm in their minute seeds, severely bottlenecks natural propagation. Consequently, determining the optimal *in vitro* conditions is paramount not only for species rescue but also for the sustainable provision of phytochemicals (Teixeira da Silva *et al.* 2015) [23].

The present study documented variable germination responses of *D. farmeri* seeds across different nutrient matrices, highlighting the profound physiological impact of basal media composition. The unequivocal superiority of the MS medium, which triggered an 83.34% germination rate is attributable to its distinct macronutrient profile. MS medium is characterized by elevated concentrations of both

ammonium (NH₄⁺) and nitrate (NO₃⁻) ions compared to KC, PM or MVW media. The immediate availability of these nitrogenous compounds provides the essential biochemical precursors necessary for rapid amino acid and protein biosynthesis during the high-energy demands of early embryogenesis and cell division.

Furthermore, the differential sucrose concentration played a critical physiological role. The MS medium in this study was fortified with 3% (w/v) sucrose, compared to the 2% utilized in the other media. Because developing orchid embryos lack endosperm, they are entirely heterotrophic and highly sensitive to external carbon sources (Chen and Chang 2000) [5]. The higher carbohydrate supply (3%) in the MS medium functioned not only as an enriched carbon skeleton for metabolic energy but also regulated the osmotic

potential of the culture environment. This precise osmotic balance is crucial for facilitating cellular expansion and the morphological transition from a swollen seed to a fully developed, photosynthetically active greenish PLBs. Conversely, the KC medium, which lacks complex organic additives and contains lower overall ionic strength and 2% sucrose, yielded the poorest germination rate (41.67%) and pale yellowish-white PLBs, indicating mild nutritional stress and delayed chlorophyll biosynthesis. These findings resonate with the observations of Bhattacharyya *et al.* (2016) [4], who similarly reported enhanced *in vitro* regeneration in the medicinal orchid *Dendrobium nobile* utilizing enriched MS formulations.

Beyond physiological propagation, the qualitative phytochemical screening underscores the pharmacological viability of this conservation protocol. Plant secondary metabolites, including alkaloids, flavonoids and terpenoids act as critical defense mechanisms in nature and serve as the foundation of traditional ethno-medicine (De 2022). A frequent concern regarding *in vitro* propagation is that the absence of natural biotic and abiotic stressors might down-regulate the biosynthetic pathways responsible for these therapeutic compounds. However, our comparative analyses revealed that *in vitro* derived plantlets of *D. farmeri* successfully retained substantial quantities of key secondary metabolites.

The spot tests indicated high (+++) responses for alkaloids, terpenoids and quinine in the *in vitro* tissues levels structurally comparable to those observed in wild harvested pseudobulbs and leaves. While the *in vitro* plantlets exhibited slightly lower concentrations of flavonoids compared to natural leaves, the retention of a broad spectrum of active compounds (including saponins, tannins and steroids) confirms that the genetic capacity for secondary metabolism remains robust under controlled conditions. This biochemical preservation suggests that the precursors and enzymatic activities governing these pathways are either inherently stable or adequately supported by the nutrient-rich *in vitro* environment. Similar phytochemical retention has been documented in other commercially valuable orchids; for instance, the synthesis of phenanthrene derivatives and alkaloids has been proven stable in tissue cultured *Dendrobium* species intended for pharmaceutical use (Bhattacharyya *et al.* 2016, Hossain 2011) [4, 10].

The complete absence of anthraquinones in both the wild and *in vitro* *D. farmeri* samples is a notable chemotaxonomic marker, consistent with profiling studies of various other *Dendrobium* species native to the Indo-Burma biodiversity hotspot. While this study was limited to qualitative spot assessments, the distinct biochemical mapping lays a critical foundation for future quantitative high performance liquid chromatography (HPLC) analyses. Ultimately, the ability to rapidly mass propagate *D. farmeri* while concurrently sustaining its medicinal phytochemical profile presents profound implications. It offers a scalable, controlled mechanism to supply the commercial pharmacological demand for *Dendrobium* extracts, thereby decisively reducing the destructive anthropogenic harvesting pressures currently driving the species toward local extinction in the forests of Bangladesh.

Conclusion

The present study successfully established a highly efficient *in vitro* asymbiotic seed germination protocol for the

endangered medicinal orchid *Dendrobium farmeri*, finding MS medium fortified with 3% sucrose as the optimal matrix for maximum percentage of germination. Crucially, comparative phytochemical profiling validated that the *in vitro* propagated plantlets actively biosynthesize and retain critical therapeutic secondary metabolites including alkaloids, terpenoids and quinine at levels comparable to naturally grown counterparts. This demonstrates that *in vitro* propagation is not only a vital conservation strategy for rescuing wild *D. farmeri* populations but also a highly sustainable and commercially viable alternative for the continuous production of pharmaceutical compounds.

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