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In vitro seed germination and phytochemical screening of *Dendrobium Crepidatum* Lindl. & Paxton, a medicinal orchid species of Bangladesh

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Abstract
Dendrobium crepidatum Lindl. & Paxton is a medicinally significant, epiphytic orchid native to Bangladesh, currently facing severe population decline due to habitat destruction and unregulated over collection. This study aimed to establish a reliable protocol for *in vitro* mass propagation and to comparatively evaluate the phytochemical profiles of naturally grown versus *in vitro* derived plantlets. Asymbiotic seed germination was tested on four basal media (KC, MS, MVW and PM), with Phytamax (PM) medium yielding the highest germination frequency (58.34%). After seedlings development successful acclimatization were completed in outside environment with a 80% survival rate. Phytochemical screening evaluated secondary metabolites, confirming the abundant presence of alkaloids, flavonoids, phenols and terpenoids across various tissues. *In vitro* derived plantlets displayed a robust secondary metabolite profile remarkably comparable to wild ecotypes. *In vitro* plantlets exhibited phytochemical profiling and validating plant tissue culture as a sustainable alternative for the commercial propagation and *ex situ* conservation of this near threatened therapeutic orchid species.

Keywords: *Dendrobium Crepidatum*, asymbiotic seed germination, acclimatization, phytochemical screening, secondary metabolites

Introduction
The family *Orchidaceae*, comprising roughly 10% of all angiosperms, represents one of the most evolutionarily advanced and morphologically diverse plant families in the world. Beyond their unparalleled horticultural and ornamental appeal, orchids possess profound therapeutic attributes, rendering them highly valuable in traditional and modern medicine [1]. In Bangladesh, the agro-climatic conditions are highly conducive to orchid vegetation, supporting around 188 species across 72 genera [2]. However, anthropogenic pressures, high deforestation rates and indiscriminate harvesting for national and international trade have restricted numerous species to narrow ecological pockets, pushing them toward the endangered category [3]. Because of their complex life histories and specialized ecological niches, orchids are thought to be particularly vulnerable to the effects of global environmental change [4].
Dendrobium, one of the largest genera within *Orchidaceae*, is heavily exploited globally for both its floricultural charm and extensive medicinal properties. *Dendrobium crepidatum* Lindl. & Paxton, an epiphytic orchid primarily distributed in the Chittagong Hill Tracts of Bangladesh, is renowned for its therapeutic significance. Traditionally, the pseudobulbs, stems and leaves of this species have been utilized in Traditional Chinese Medicine (TCM) and local herbal remedies for treating bone fractures, diabetes, cataracts and fever. Pharmacological studies highlight its notable neuroprotective, anti-inflammatory and immunomodulatory properties [5, 6].
Due to its complex life history, natural propagation of *D. crepidatum* is severely limited. Orchid seeds are microscopic, lack endosperm and obligately depend on species-specific mycorrhizal fungal associations to supply essential carbohydrates and nutrients for germination in the wild [7]. Vegetative propagation is excessively slow and insufficient to meet the commercial pharmaceutical demand. To mitigate the risk of extinction and support sustainable commercial exploitation, biotechnological interventions, particularly *in vitro* plant tissue culture, offer a viable solution. Asymbiotic seed germination and micropropagation utilizing somatic tissues (e.g., nodal and leaf segments) *via* protocorm-like bodies (PLBs) ensure the rapid mass multiplication of desired clones [8]. While scattered reports exist regarding the *in vitro* culture of various *Dendrobium* species, optimization is highly species specific.
Plants synthesize a vast array of secondary metabolites (e.g., alkaloids, flavonoids, tannins and phenolics) that serve as defense mechanisms against herbivores and microorganisms and offer significant pharmacological benefits to humans [9, 10]. In medicinal orchids, alkaloids such as dendrobine and various phenolic compounds act as the primary bioactive constituents. A major challenge in plant tissue culture is the potential loss of biochemical fidelity; *in vitro* environments can sometimes alter the biosynthetic pathways of secondary metabolites [11]. Ensuring that *in vitro* propagated orchids retain these bioactive constituents is crucial for their long-term pharmaceutical application.
Therefore, the present study was undertaken to: (a) develop a highly efficient and reproducible protocol for the *in vitro* asymbiotic seed germination and mass propagation of *D. crepidatum*, (b) successfully acclimatize the regenerated seedlings and (c) conduct an extensive comparative evaluation of the phytochemical profiles between naturally grown plants and *in vitro* derived plantlets.

Materials and Methods
1. Plant Material and Sterilization
Mature, un-dehisced capsules and vegetative parts (leaves, stems and roots) of naturally grown *Dendrobium crepidatum* were collected from Remakri, Bandarban, Bangladesh. For *in vitro* culture, the capsules were thoroughly washed under running tap water to remove superficial dust, followed by a wash with sterile distilled water. The capsules were then surface sterilized in a laminar airflow cabinet using a 0.2% (w/v) mercuric chloride

($HgCl_2$) solution for 5 minutes, followed by 3-4 rinses in sterile double distilled water. A final disinfection was performed using 70% ethanol for 1 minute, followed by an additional 2-3 rinses with sterile distilled water.

2. Media Preparation for Asymbiotic Seed Germination

Four solid basal media were evaluated for seed germination efficiency: KC (Knudson 1946) [12], MS (Murashige and Skoog 1962) [13], MVW (Vacin and Went 1946) [14] and PM: Phytamax (Arditti 1977) [15]. The MS medium was supplemented with 3% (w/v) sucrose, while KC, MVW and PM contained 2% (w/v) sucrose. All media were solidified with 0.8% (w/v) agar. The pH was adjusted to 5.8 for MS and to respective specific requirements for the other media (5.4 for PM and MVW, 5.0 for KC) using 1N NaOH or 1N HCl before autoclaving at 121°C for 20 minutes. The sterilized capsules were longitudinally dissected and the powdery seeds were aseptically inoculated onto the culture media. Cultures were incubated at 25 ^{0}C under a 14-hour photoperiod provided by cool white fluorescent tubes.

3. Acclimatization

Well-rooted *in vitro* seedlings were removed from the culture vessels, gently washed under running tap water to remove adhering agar and subjected to sequential acclimatization. Seedlings were initially exposed to room conditions by opening the culture vessels, followed by transfer to small earthen pots containing a moistened mixture of coconut coir, coir dust and coal. They were maintained at room temperature with high humidity for initial establishment before transferring to a greenhouse environment.

4. Phytochemical Screening

Fresh leaf, stem and root tissues from naturally grown orchids, alongside *in vitro* regenerated tissues (callus, shoot buds, SPSs and complete plantlets), were evaluated for secondary metabolites. For alkaloid spot tests, 5g of finely chopped fresh plant material was moistened with 10 ml of 2% HCl and heated in a water bath at 60°C for one hour before filtration. For the remaining metabolites, methanolic crude extracts were prepared by macerating the dried powder in methanol, heating at 60°C and subsequently evaporating the solvent.

Alkaloids: Assessed using five distinct reagents: Dragendorff's (bismuth nitrate), Hager's (picric acid), Mayer's (mercuric chloride), Wagner's (iodine/potassium iodide) and Tannic acid.

Other Metabolites: Standard biochemical assays were conducted for phlobatannins (1% aqueous HCl), flavonoids (ethyl acetate and ammonia), saponins (froth test), tannins (ferric chloride), terpenoids and steroids (chloroform and concentrated sulfuric acid), glycosides (Fehling's solution), anthraquinones (magnesium acetate), quinine, coumarin, phenols and proteins.

Scoring: The relative abundance of precipitate or color change was recorded visually as '+++' (heavy/substantial), '++' (moderate), '+' (slight), or '-' (absent).

Results

1. Asymbiotic Seed Germination

Seeds of *D. crepidatum* successfully germinated across all tested media, though germination frequencies varied significantly depending on the nutritional composition (Table 1). The highest germination rate was recorded on the PM medium (58.34%, Fig. 1), characterized by the rapid development of robust, greenish-yellow PLBs. This was followed by MS medium (50.00%, Fig. 2) and MVW medium (33.34%, Fig. 3). The KC medium exhibited the lowest germination competency (25.00%). Germinated PLBs subsequently developed into tiny seedlings upon subculturing into fresh media at a lower density.

Table 1: *In vitro* germination of seeds of *D. crepidatum* Lindl. & 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	25.00	Yellowish white	+
MS	3% (w/v) sucrose	12	06	50.00	Greenish	++
PM	2% (w/v) sucrose	12	07	58.34	Greenish yellow	++
MVW	2% (w/v) sucrose	12	04	33.34	Yellowish green	+

*Each experiment consists of 12 replicates.

2. Acclimatization

Rooted seedlings were transferred *ex vitro via* a gradual hardening process. Seedlings potted in a composite substrate of moist coconut coir, sawdust and coal demonstrated a 80% survival rate (Fig. 4). The established seedlings displayed healthy vegetative growth, developing new leaves after 2-3 months in the greenhouse environment.

Fig 1: Seed germination of *Dendrobium crepidatum* on PM medium

Fig 2: Seed germination of *D. crepidatum* on MS medium

Fig 3: Seed germination of *D. crepidatum* on MVW medium

Fig 4: *In vitro* developed *D. crepidatum* seedling growing in pot outside of the culture room

3. Phytochemical Profiling

Extensive qualitative phytochemical screening of methanolic and acidic extracts from naturally grown plant parts (leaf, stem, root) and *in vitro* derived tissues revealed a rich repository of secondary metabolites, highlighting significant tissue-specific variations.

Alkaloids: High concentrations (+++) of alkaloids were detected prominently in the natural stem and the *in vitro* shoot buds. Among the five reagents tested, Wagner's reagent demonstrated the highest sensitivity, yielding heavy (+++) precipitate responses across virtually all tissue types (natural and *in vitro*). Dragendorff's and Hager's reagents also indicated heavy presence (+++) in the natural leaf and stem, as well as in *in vitro* shoot buds and SPSs (Shoot Primordia-Like Structures). This comprehensive profiling indicates that *in vitro* conditions did not suppress secondary metabolism but rather supported a robust phytochemical profile functionally equivalent to wild ecotypes (Table 2).

Table 2: Qualitative test for alkaloids of natural and *in vitro* grown sample of *Dendrobium crepidatum*

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

Notes: 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.

Other Metabolites

Natural Leaf: Exhibited the highest (+++) phenolic content and a moderate (++) presence of phlobatannins, flavonoids, terpenoids, steroids, quinine and proteins (Table 3). Saponins, tannins and anthraquinones were absent (-).

Natural Stem: Showed profound accumulation (+++) of flavonoids, terpenoids, steroids, glycosides and quinine (Table 3). Saponins and anthraquinones were completely absent (-).

Natural Root: Demonstrated the highest abundance (+++) of flavonoids, tannins, terpenoids, steroids, quinine and coumarin (Table 3). Glycosides and saponins were absent (-).

***In vitro* Plantlets:** Crucially, the *in vitro*-regenerated plantlets synthesized substantial quantities (+++) of phlobatannins, quinine, tannins, coumarin and phenols. They also exhibited moderate (++) levels of flavonoids, terpenoids, glycosides and steroids (Table 3). Saponins and anthraquinones were uniformly absent (-).

Table 3: Qualitative test of twelve secondary metabolites of natural and *in vitro* developed plantlets of *Dendrobium crepidatum* Lindl. and Paxton

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

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

Discussion

The establishment of an efficient micropropagation protocol is critical for the conservation and commercial utilization of endangered medicinal orchids like *Dendrobium crepidatum*. In our study, the superiority of the PM (Phytamax) medium for asymbiotic seed germination (58.34%) can be attributed

to its specialized nutrient formulation enriched with organic supplements tailored for orchid species. The nutritional requirements for orchid seed germination vary significantly based on their physiological state and symbiotic dependencies [7]. Similar enhancements in germination competency using targeted basal media have been reported for *Robiquetia spathulata* and *Dendrobium fimbriatum* [16, 17]. Root induction was optimally achieved which effectively stimulated adventitious root primordial a crucial step ensuring a high *ex vitro* survival rate during acclimatization.

The phytochemical fidelity of tissue cultured plants is a primary concern in pharmaceutical applications. Environmental stressors in the wild often trigger the accumulation of defense compounds (secondary metabolites), which can sometimes be down regulated in nutrient rich *in vitro* environments [11]. However, our screening confirmed the abundant presence of critical metabolites, particularly alkaloids, in both natural stems and *in vitro* shoot buds. This aligns with recent findings by Wang (2021) [18], who reported a high yield of total alkaloids from the stems of *D. crepidatum* [18]. Alkaloids in *Dendrobium* (e.g., dendrobine) are responsible for notable neuroprotective, analgesic and antipyretic effects in Traditional Chinese Medicine [5]. The robust accumulation of phenols, flavonoids, tannins and coumarins in the *in vitro* plantlets explicitly validates that tissue culture environments successfully sustain the complex biosynthetic pathways of these secondary metabolites [19]. The uniform absence of saponins and anthraquinones across nearly all tissues confirms a conserved metabolic signature for this species.

Conclusion

This study successfully developed a highly efficient, reliable and rapid seed germination protocol and subsequent seedlings development and acclimatization in outside environment. Crucially, extensive qualitative phytochemical screening demonstrated that *in vitro* regenerated plantlets retain a rich secondary metabolite profile highly comparable to their wild counterparts. This validates the use of biotechnological interventions not only as a critical tool for the *ex situ* conservation of this rare species but also as a sustainable, commercial platform for the continuous production of vital pharmaceutical compounds without exploiting delicate natural habitats.

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