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Establishment of an efficient *in vitro* seed germination and micropropagation protocol for *Dendrobium aggregatum* Roxb.: A medicinally important orchid of Bangladesh

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Abstract
Dendrobium aggregatum Roxb. is an epiphytic medicinal and ornamental orchid indigenous to the Chittagong Hill Tracts of Bangladesh. Due to habitat destruction, over exploitation and the species' intrinsic biological limitations specifically the lack of endosperm and absolute dependency on mycorrhizal fungi for natural seed germination wild populations are rapidly declining. This study was undertaken to establish a reproducible, highly efficient *in vitro* seed germination and direct micropropagation protocol for the conservation and mass multiplication of *D. aggregatum*. Immature capsules were aseptically cultured on four basal media formulations: Knudson C (KC), Murashige and Skoog (MS), Phytamax (PM) and Modified Vacin and Went (MVW), evaluated with and without plant growth regulators (PGRs). Full strength MS medium supplemented with 0.5 mg/l 6-Benzylaminopurine (BAP) and 0.5 mg/l α-Naphthaleneacetic acid (NAA) exhibited absolute superiority, yielding a 100.00% germination rate and initiating early protocorm development within 10.13 ± 0.41 weeks. Subsequent complete plantlet development occurred in 25.53 ± 0.56 weeks. For rapid shoot elongation, transferring germinated seedlings to MS medium fortified with 1.5 mg/l Kinetin (Kn) and 0.9 mg/l Indole-3-acetic acid (IAA) induced a maximum longitudinal increase of 2.88 ± 0.04 cm over 30 days. High frequency micropropagation *via* direct organogenesis was optimally achieved using nodal segments on MS medium enriched with 2.0 mg/l BAP and 1.0 mg/l NAA, delivering 72.47 ± 1.56% response and 4.8 ± 0.42 multiple shoot buds (MSBs) per explant in 8.77 ± 0.18 weeks. Conversely, indirect morphogenic responses via protocorm-like bodies (PLBs) were optimally induced from leaf segments using 2.0 mg/l BAP and 1.0 mg/l IAA (50.00 ± 1.43%). Vigorous rhizogenesis was obtained on MS medium utilizing a synergistic combination of 1.0 mg/l Indole-3-butyric acid (IBA) and 1.0 mg/l NAA, yielding an average of 5.25 ± 0.37 roots per shoot with a mean length increase of 3.43 ± 0.05 cm. Following sequential *ex vitro* acclimatization in a substrate mix of coir, sawdust, coal, small bricks and activated charcoal, the seedlings demonstrated a robust 80% survival rate. This optimized protocol provides a reliable framework for the largescale commercial cultivation and *ex situ* conservation of *D. aggregatum*.

Keywords: Acclimatization, asymbiotic germination, *Dendrobium Aggregatum*, micropropagation, protocorm-like bodies (plbs), rhizogenesis

Introduction

The Orchidaceae family, representing one of the largest and most evolved lineages of flowering plants, comprises over 35,000 recognized species distributed across diverse global ecosystems (Chase *et al.* 2015, Hawkes 1978, Schultes and Pease 1963) [7, 11, 30]. Beyond their profound aesthetic and floricultural dominance, orchids have been recognized for millennia for their complex secondary metabolism and medicinal efficacy, frequently serving as the foundational botanical components in Ayurvedic and traditional Chinese pharmacopoeias (Nongdam 2014, Okhale *et al.* 2014, Pant *et al.* 2022) [22, 23, 24]. *Dendrobium aggregatum* Roxb., a prominent epiphytic species characterized by its pendulous inflorescences bearing vibrant, fragrant yellow flowers, is indigenous to the tropical and subtropical forests of Southeast Asia, including the Chittagong Hill Tracts (CHT) of Bangladesh (Hossain 2013, Huda *et al.* 2006) [12, 13]. Economically, *D. aggregatum* is highly prized in the global floriculture industry as a premier cut flower and potted ornamental. Medicinally, the genus *Dendrobium* is a rich source of tonic, astringent, analgesic and anti-inflammatory biomolecules utilized to treat gastrointestinal disorders, fevers and neurodegenerative conditions (Chen and Chen 1935, Hossain 2013, Singh *et al.* 2012) [8, 12, 31].

Despite its immense economic and therapeutic value, wild populations of *D. aggregatum* in Bangladesh are under severe threat. The localized extinction risk is driven primarily by rampant deforestation, anthropogenic habitat degradation and unsustainable harvesting for the illicit horticultural trade (Bhowmik and Rahman 2020, Huda *et al.* 2006) [6, 13]. Furthermore, the natural regeneration of *D. aggregatum* is severely restricted by the species' intrinsic biological architecture. Orchid seeds are microscopically minute (0.5-2.0 mm), utterly lack endosperm reserves and possess anatomically disorganized embryos (Arditti 1992, Thompson 1977) [4, 33]. Consequently, natural germination is exceptionally rare and relies entirely on a highly specific, symbiotic relationship with compatible mycorrhizal fungi in the host microenvironment to provide initial metabolic carbohydrates (Rasmussen and Rasmussen 2014, Targu *et al.* 2023) [14, 32].

To circumvent these reproductive bottlenecks and alleviate the intense pressure on wild populations, *in vitro* plant tissue culture techniques offer a crucial intervention (Arditti and Ernst 1993, Chugh *et al.* 2009, Goh 1982) [2, 9, 10]. Asymbiotic seed germination and micropropagation protocols not only ensure rapid, high frequency mass multiplication of diseasefree clones but also serve as

foundational strategies for robust *ex situ* conservation (Bhowmik and Rahman 2021, Kauth *et al.* 2006) [5, 16]. While previous studies have explored the *in vitro* morphogenic potential of various *Dendrobium* species such as *D. primulinum* (Pant and Thapa 2012) [25], *D. palpebrae* (Bhowmik and Rahman 2020) [6] and *D. chrysotoxum* (Kaur 2017) [15] the distinct morphogenic recalcitrance and nutrient specificities of *D. aggregatum* demand a highly tailored protocol. The efficacy of primary media compositions such as Murashige and Skoog (MS), Knudson C (KC), Phytamax (PM) and Modified Vacin and Went (MVW) varies drastically across orchid taxa, heavily modulated by precise exogenously applied plant growth regulators (PGRs) matrices encompassing auxins and cytokinins (Jain 1997, Naing *et al.* 2011, Reddy *et al.* 2021) [14, 21, 29].

The primary objective of the present investigation was to systematically establish a dependable, efficient and reproducible protocol for the largescale *in vitro* seed germination and micropropagation of *Dendrobium aggregatum* Roxb. Specifically, this study evaluates the differential morphogenic impacts of various basal media on seed germination kinetics, optimizes the cytokinin auxin ratios necessary for maximum shoot elongation and high frequency multiple shoot buds (MSBs) induction and defines the precise hormonal requisites for robust *in vitro* rhizogenesis and subsequent *ex vitro* acclimatization.

Materials and Methods

1. Plant Material Collection and Surface Sterilization

Immature, green, un-dehisced capsules of *Dendrobium aggregatum* Roxb. were collected from naturally growing wild populations in the Chittagong Hill Tracts (CHT), Bangladesh. To eliminate epiphytic contaminants and surface debris, the capsules were thoroughly scrubbed under running tap water for 20 minutes, followed by a vigorous wash in a mild detergent solution supplemented with 2-4 drops of Tween-20 for 15 minutes and rinsed thoroughly with distilled water. All subsequent sterilization steps were executed under strict aseptic conditions within a laminar airflow cabinet. The capsules were submerged in a 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10 minutes, rinsed sequentially in 70% (v/v) ethanol for 30 seconds and finally subjected to 3-4 consecutive washes with sterile double distilled water to remove all traces of chemical sterilants. The surface sterilized capsules were air-dried on sterile double layer Whatman filter paper to absorb residual moisture.

2. Media Preparation for Seed Germination

Four distinct basal media formulations were evaluated for initial asymbiotic seed germination: Knudson C: KC (Knudson 1946) [17], Murashige and Skoog: MS (Murashige and Skoog 1962) [20], Phytamax: PM (Arditti 1977) [3] and Modified Vacin and Went: MVW (Vacin and Went 1949) [34]. Media were prepared using analytical grade stock solutions. Sucrose was incorporated as the primary carbon source at a concentration of 3% (w/v) for the MS medium and 2% (w/v) for the KC, PM and MVW media. To solidify the formulations, 0.8% (w/v) agar (Hi Media, India) was utilized. The media were evaluated both with or without the addition of Plant Growth Regulators (PGRs free control) and with a standardized PGRs supplementation of 0.5 mg/l 6-Benzylaminopurine (BAP) and 0.5 mg/l α-Naphthalene acetic acid (NAA). The pH of the MS medium was digitally adjusted to 5.8, while the KC, PM and MVW media were adjusted to 5.4 using 1N NaOH or 1N HCl prior to agar addition. Aliquots of 30-50 ml of the media were dispensed into appropriate glass culture vessels (150 ml screw capped bottles or 2.5 cm × 15 cm test tubes) and autoclaved at 121°C and 1.9 kg/cm² pressure for 20 minutes.

3. Asymbiotic Seed Inoculation and Culture Conditions

The sterilized capsules were longitudinally dissected on a sterile aluminum tile using a sterile surgical scalpel. The minute, powdery seeds were aseptically scooped and dispersed uniformly across the surface of the gelled media. All culture vessels were subsequently transferred to a controlled growth room maintained at a temperature of 25 ± 2°C with a relative humidity of 50-60%. A 14-hour photoperiod consisting of 4000-5000 lux illumination provided by cool white fluorescent tubes, alternated with a 10-hour dark phase, was strictly maintained to facilitate protocorm development and primary differentiation. Sub-culturing of the developing protocorms was executed at 30-35day intervals to prevent nutrient depletion and density induced necrosis.

4. Protocol for Shoot Elongation and Micropropagation

To induce rapid inter-nodal elongation, seed derived early plantlets (1-2 leaves) were transferred to full strength MS media supplemented with varying concentrations and combinations of cytokinins (BAP, Kn) and auxins (NAA, IAA). For mass micropropagation, established *in vitro* seedlings (5-6 cm in height) were utilized as explant sources. Nodal segments (1.0-1.5 cm) and leaf segments (0.5-1.0 cm) were aseptically excised and inoculated onto full strength MS media fortified with twentyfive different PGRs configurations to evaluate the induction frequency of multiple shoot buds (MSBs) *via* direct organogenesis and protocorm-like bodies (PLBs) *via* indirect embryogenesis. Sub-culturing was carried out systematically every 25-30 days.

5. *In vitro* Rhizogenesis and *Ex vitro* Acclimatization

Elongated micro-shoots (2-3 cm) were excised and transferred to nineteen distinct rooting formulations consisting of fullstrength MS basal medium supplemented with variable concentrations of auxins (IAA, IBA, NAA) to induce a robust root architecture. Following the establishment of a well-differentiated root system, the seedlings underwent a sequential acclimatization protocol. Culture vessel caps were loosened for 24 hours within the growth room to initiate ambient exposure, followed by a 6-hour and then 12-hour external acclimatization phase. Seedlings were subsequently extracted, roots were thoroughly washed under running tap water to remove all agar residues and they were transferred into small pots containing a specialized epiphytic substrate composed of small bricks, activated charcoal, wooden coal, sawdust and coconut husk at a volumetric ratio of 1:1:1:1:1. The substrate was pre-treated with 0.1% Agrosan fungicide. After 3-5 days of high humidity ambient adaptation, the orchids were transferred to a greenhouse environment (25-30°C, RH 60-70%) for permanent establishment.

6. Experimental Design and Statistical Analysis

All tissue culture experiments were structured using a Completely Randomized Design (CRD). Seed germination

treatments utilized 15 replicates per formulation, while micropropagation and rooting experiments utilized 5 and 6 replicates per treatment, respectively. Growth kinetics, including the percentage of responding explants, timeline for developmental stages (measured in weeks), number of multiple shoots/ roots and elongation metrics (cm), were rigorously documented. Data are expressed as the Mean ± Standard Error (SE) and were computed using Microsoft Excel 2013 software.

Results

1. *In vitro* Seed Germination and Morphogenesis

The asymbiotic germination of *D. aggregatum* seeds was critically influenced by both the chemical composition of the basal media and the incorporation of exogenous plant growth regulators. As documented in Table 1, swelling of the embryos and the subsequent developmental cascade (protocorm formation, leaf primordia differentiation, root primordia induction and complete seedling maturation) were observed across all tested media (KC, MS, PM, MVW), albeit with highly variable efficiencies.

The application of fullstrength MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA exhibited absolute superiority, yielding a 100.00% germination rate on the culture vessels (Fig. 1). This optimal configuration drastically accelerated the morphogenic timeline; initial seed germination was observed in just 8.17 ± 0.40 weeks, followed by the rapid development of distinctive green protocorms at 10.13 ± 0.41 weeks (Fig. 2). Subsequent differentiation proceeded seamlessly, with first leaf primordia emerging at 14.31 ± 0.52 weeks and first root primordia at 19.62 ± 0.53 weeks. The complete, fully differentiated plantlets were achieved within a minimal timeframe of 25.53 ± 0.56 weeks (Fig. 3).

Full strength PM medium supplemented with PGRs demonstrated the second highest efficacy, supporting a 91.67% germination rate and requiring 26.25 ± 0.57 weeks for complete seedling development. Conversely, PGRs free half strength KC medium proved to be the least optimal environment, supporting a poor germination rate of 33.34% and prolonging complete plantlet maturation to a protracted 36.13 ± 0.47 weeks. The data explicitly underscore the necessity of fullstrength MS nutrients combined with balanced auxin/cytokinin ratios for the optimized morphogenic transition of *D. aggregatum* embryos.

2. Shoot Elongation Optimization

Following primary germination, the resulting miniature shoots required specific hormonal stimulation to achieve robust longitudinal extension prior to micropropagation. As summarized in Table 2, seed derived plantlets exhibited a highly significant elongation response upon exposure to synergistic cytokinin and auxin matrices over a 30-days culture period. The absolute highest rate of longitudinal extension was achieved on MS medium fortified with 1.5 mg/l Kn and 0.9 mg/l IAA (Fig. 4). Plantlets on this formulation increased from a baseline length of 1.37 ± 0.03 cm to a final length of 4.25 ± 0.06 cm, registering a net mean increase of 2.88 ± 0.04 cm. This was closely followed by the combination of 2.0 mg/l Kn + 1.2 mg/l NAA, which facilitated a net increase of 2.79 ± 0.02 cm. The empirical data dictates that Kinetin (Kn) functionally combined with IAA is decidedly superior to BAP-NAA combinations for promoting rapid inter-nodal elongation in this specific species.

3. Micropropagation (Direct Organogenesis and Embryogenesis)

To facilitate large scale multiplication, *in vitro* raised nodal and leaf segments were subjected to twentyfive discrete PGRs matrices on full strength MS media. The morphogenic pathway strictly varied by explant type: nodal segments bypassed an intermediate stage and engaged in direct organogenesis *via* producing multiple shoot buds (MSBs), whereas leaf segments engaged in indirect embryogenesis *via* producing protocorm-like bodies (PLBs).

As detailed in Table 3, nodal segments exhibited the maximum prolific capability on MS medium fortified with 2.0 mg/l BAP and 1.0 mg/l NAA. Under this regimen, an outstanding 72.47 ± 1.56% of explants responded, producing an average of 4.8 ± 0.42 MSBs per nodal segment within a remarkably brief induction period of 8.77 ± 0.18 weeks (Fig. 5). Substituting NAA with Picloram (2.0 mg/l BAP + 1.0 mg/l Pic) proved to be the second most effective matrix, yielding a 65.67 ± 1.92% induction rate and 3.9 ± 0.71 MSBs per explant in 9.76 ± 0.14 weeks.

Conversely, leaf explants responded optimally to MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l IAA. This formulation triggered cellular reprogramming, leading to the formation of dense, greenish PLBs in 50.00 ± 1.43% of the cultures within 9.42 ± 0.13 weeks (Fig. 6). A secondary optimal response for PLBs induction was noted at 47.00 ± 2.32% on MS + 2.0 mg/l BAP + 1.0 mg/l NAA (10.34 ± 0.13 weeks). Control treatments devoid of PGRs failed to induce any MSBs or PLBs across both explant types, affirming the critical dependency on exogenous PGRs signaling for somatic tissue proliferation.

4. Rooting Architecture and Rhizogenesis

The transition from *in vitro* proliferation to *ex vitro* acclimatization requires the formation of a robust, physiologically functional root system. The isolated micro-shoots were cultured on nineteen different auxinbased MS rooting matrices (Table 4). The absolute highest efficiency in rhizogenesis marked by both the quantity and length of roots was achieved on MS medium evenly supplemented with 1.0 mg/l IBA and 1.0 mg/l NAA. Over 30 days, this specific formulation induced a mean of 5.25 ± 0.37 roots per shoot, alongside a significant primary root length increase of 3.43 ± 0.05 cm (Fig. 7) followed by the combination of 1.0 mg/l IAA + 1.0 mg/l IBA also proved highly effective, generating 5.08 ± 0.21 roots per shoot with a longitudinal increase of 3.09 ± 0.06 cm. The data conclusively demonstrate that a synergistic interaction between IBA and NAA is vastly superior to single-auxin treatments for stimulating robust root architecture in *D. aggregatum*.

5. Acclimatization and Transplantation

In vitro generated *D. aggregatum* seedlings possessing 4-5 vigorous leaves and a densely ramified root system were sequentially transitioned to ambient conditions. Upon washing and transferring to the optimized porous substrate matrix (coir, sawdust, coal, small bricks and activated charcoal), the seedlingss rapidly adapted. The gradual decrease in relative humidity combined with fungicide protection allowed the seedlingss to initiate new *ex vitro* foliar and root growth within 4 weeks. Ultimately, a survival rate of 80% was achieved (Fig. 8), confirming the physiological integrity and structural vigor of the micro-propagated clones.

Table 1: Effect of different strength of KC, MS, PM and MVW media with or without PGRs on *in vitro* seed germination, differentiation and seedlings development of *Dendrobium aggregatum* Roxb.

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 leafprimordia (Mean ± SE)	Differentiation of 1st root primordia (Mean ± SE)	Development of seedlings (Mean ± SE)		
KC	Half without PGRs	15.34 ± 0.28	19.07 ± 0.51	24.22 ± 0.55	28.71 ± 0.40	36.13 ± 0.47	33.34	+
	Full without PGRs	13.49 ± 0.29	16.05 ± 0.52	21.82 ± 0.55	26.95 ± 0.41	33.09 ± 0.45	41.67	+
	Full with PGRs	11.23 ± 0.36	13.98 ± 0.61	18.86 ± 0.48	22.90 ± 0.48	28.87 ± 0.41	66.67	++
MS	Half without PGRs	11.17 ± 0.33	15.33 ± 0.45	19.84 ± 0.48	26.41 ± 0.43	30.23 ± 0.49	73.34	++
	Full without PGRs	9.42 ± 0.31	12.26 ± 0.47	17.48 ± 0.46	24.37 ± 0.45	27.17 ± 0.47	83.34	+++
	Full with PGRs	8.17 ± 0.40	10.13 ± 0.41	14.31 ± 0.52	19.62 ± 0.53	25.53 ± 0.56	100.00	+++
PM	Half without PGRs	13.12 ± 0.36	15.58 ± 0.48	20.47 ± 0.61	25.11 ± 0.43	30.67 ± 0.47	66.67	++
	Full without PGRs	10.08 ± 0.35	13.07 ± 0.46	18.32 ± 0.59	22.38 ± 0.44	28.04 ± 0.49	80.00	+++
	Full with PGRs	8.76 ± 0.29	11.89 ± 0.59	15.87 ± 0.47	20.74 ± 0.56	26.25 ± 0.57	91.67	+++
MVW	Half without PGRs	17.14 ± 0.39	18.65 ± 0.37	24.04 ± 0.65	29.33 ± 0.53	35.05 ± 0.54	46.67	+
	Full without PGRs	14.92 ± 0.38	16.25 ± 0.38	21.14 ± 0.64	26.87 ± 0.53	32.08 ± 0.52	58.34	++
	Full with PGRs	12.13 ± 0.33	14.32 ± 0.53	18.50 ± 0.51	23.49 ± 0.40	27.97 ± 0.51	83.34	+++

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 15 replicates

Table 2: Elongation of *in vitro* germinated seedlings of *D. aggregatum* on 0.8% (w/v) agar solidified full strength MS medium with different kinds of PGRs.

Sl. No.	PGR Conc. (mg/l)				Solid media		
	BAP	Kn	NAA	IAA	Initial length (cm) of seedlings aftergermination (Mean±SE)	Length (cm) of seedlings after 30d of culture on elongation medium (Mean±SE)	Increased in length (cm) of seedlings within 30d of culture on elongation medium (Mean±SE)
1.	0.5	-	-	-	1.34 ± 0.04	3.29 ± 0.03	1.95 ± 0.02
2.	1.0	-	-	-	1.33 ± 0.03	2.93 ± 0.03	1.60 ± 0.02
3.	1.5	-	-	-	1.30 ± 0.01	2.87 ± 0.02	1.57 ± 0.03
4.	2.0	-	-	-	1.35 ± 0.03	3.12 ± 0.06	1.77 ± 0.05
5.	-	0.5	-	-	1.38 ± 0.02	3.55 ± 0.05	2.17 ± 0.04
6.	-	1.0	-	-	1.34 ± 0.02	3.27 ± 0.04	1.93 ± 0.03
7.	-	1.5	-	-	1.31 ± 0.04	3.18 ± 0.06	1.87 ± 0.03
8.	-	2.0	-	-	1.33 ± 0.02	3.57 ± 0.04	2.24 ± 0.02
9.	0.5	-	0.3	-	1.28 ± 0.03	3.37 ± 0.02	2.09 ± 0.02
10.	1.0	-	0.6	-	1.31 ± 0.01	3.11 ± 0.02	1.80 ± 0.02
11.	1.5	-	0.9	-	1.37 ± 0.04	3.68 ± 0.06	2.31 ± 0.04
12.	2.0	-	1.2	-	1.36 ± 0.03	3.93 ± 0.04	2.57 ± 0.03
13.	0.5	-	-	0.3	1.35 ± 0.03	3.76 ± 0.02	2.41 ± 0.04
14.	1.0	-	-	0.6	1.30 ± 0.01	3.61 ± 0.02	2.31 ± 0.04
15.	1.5	-	-	0.9	1.34 ± 0.02	3.75 ± 0.03	2.41 ± 0.02
16.	2.0	-	-	1.2	1.32 ± 0.03	2.89 ± 0.06	1.57 ± 0.03
17.	-	0.5	0.3	-	1.33 ± 0.02	3.81 ± 0.03	2.48 ± 0.04
18.	-	1.0	0.6	-	1.31 ± 0.04	3.58 ± 0.03	2.27 ± 0.04
19.	-	1.5	0.9	-	1.33 ± 0.02	3.12 ± 0.05	1.79 ± 0.03
20.	-	2.0	1.2	-	1.35 ± 0.03	4.14 ± 0.03	2.79 ± 0.02

21.	-	0.5	-	0.3	1.34 ± 0.02	3.89 ± 0.03	2.55 ± 0.03
22.	-	1.0	-	0.6	1.30 ± 0.02	4.02 ± 0.04	2.72 ± 0.03
23.	-	1.5	-	0.9	1.37 ± 0.03	4.25 ± 0.06	2.88 ± 0.04
24.	-	2.0	-	1.2	1.36 ± 0.04	3.63 ± 0.04	2.27 ± 0.03
25.	MS0 (Control)				1.32 ± 0.02	2.72 ± 0.03	1.40 ± 0.02

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

Table 3: Development of MBSs/PLBs from *in vitro* raised nodal and leaf explants of *D. aggregatum* when grown on agar solidified MS medium with different kinds of PGRs.

Sl. No.	PGRs Concentration (mg/l)					Nodal Segment (Mean ± SE)			Leaf Segment (Mean ± SE)		
	BAP	Kn	NAA	IAA	Pic	% of induced MBSs/ segment	Time required (week) for development of MSBs	No. of MBSs produced/segment	% of induced PLBs/ segment	Time required (week) for development of PLBs	Nature of PLBs (Colour)
1.	1.0	-	-	-	-	28.00±1.41	11.46±0.09	1.4±0.49	0.00±0.00	0.00±0.00	-
2.	2.0	-	-	-	-	39.33±1.37	10.72±0.14	2.1±0.67	5.67±1.19	13.36±0.15	G
3.	3.0	-	-	-	-	28.00±1.45	12.83±0.14	1.1±0.54	5.33±1.37	10.76±0.19	YG
4.	-	1.0	-	-	-	17.67±1.48	12.46±0.10	1.5±0.37	0.00±0.00	0.00±0.00	-
5.	-	2.0	-	-	-	22.33±2.31	11.78±0.18	1.8±0.55	5.67±1.27	12.18±0.17	G
6.	-	3.0	-	-	-	20.47±1.56	13.67±0.11	1.4±0.39	18.00±1.46	11.23±0.15	WG
7.	1.0	-	0.5	-	-	59.33±1.94	10.66±0.13	3.5±0.36	29.67±1.71	11.62±0.11	G
8.	2.0	-	1.0	-	-	72.47±1.56	8.77±0.18	4.8±0.42	47.00±2.32	10.34±0.13	G
9.	3.0	-	1.5	-	-	62.67±1.55	12.54±0.13	3.3±0.37	37.33±1.75	11.51±0.11	G
10.	1.0	-	-	0.5	-	53.33±1.91	10.14±0.12	3.2±0.56	41.67±1.47	11.54±0.07	WG
11.	2.0	-	-	1.0	-	58.00±1.87	10.26±0.10	3.8±0.49	50.00±1.43	9.42±0.13	G
12.	3.0	-	-	1.5	-	50.67±1.65	13.10±0.13	3.3±0.58	43.67±1.96	11.27±0.07	G
13.	1.0	-	-	-	0.5	53.33±1.89	10.34±0.16	3.5±0.53	28.33±1.61	11.74±0.13	YG
14.	2.0	-	-	-	1.0	65.67±1.92	9.76±0.14	3.9±0.71	36.00±1.68	10.29±0.19	G
15.	3.0	-	-	-	1.5	60.00±1.78	13.31±0.18	2.8±0.55	33.67±1.67	10.42±0.13	G
16.	-	1.0	0.5	-	-	36.47±1.35	12.45±0.13	1.6±0.45	12.33±1.25	12.56±0.18	WG
17.	-	2.0	1.0	-	-	42.33±1.50	10.23±0.18	2.3±0.55	17.00±1.67	10.58±0.15	G
18.	-	3.0	1.5	-	-	32.67±1.34	13.40±0.13	1.9±0.45	16.33±1.58	12.78±0.18	G
19.	-	1.0	-	0.5	-	31.00±1.49	10.57±0.15	1.2±0.67	0.00±0.00	0.00±0.00	-
20.	-	2.0	-	1.0	-	40.33±1.85	11.28±0.13	2.3±0.68	21.37±1.28	11.48±0.12	YG
21.	-	3.0	-	1.5	-	28.67±1.42	12.04±0.11	1.0±0.61	20.00±1.86	10.57±0.12	G
22.	-	1.0	-	-	0.5	37.27±1.47	11.36±0.16	1.8±0.53	0.00±0.00	0.00±0.00	-
23.	-	2.0	-	-	1.0	47.67±1.91	11.14±0.19	2.5±0.54	16.00±2.17	12.36±0.15	YG
24.	-	3.0	-	-	1.5	35.33±1.38	12.45±0.17	1.6±0.58	18.33±2.19	10.63±0.15	G
25.	MS0 (Control)					10.00±1.38	12.36±0.14	1.2±0.18	0.00±0.00	0.00±0.00	-

Multiple Shoot Buds (MBSs); Protocorm Like Bodies (PLBs); '-' Indicate no response; Greenish PLBs (G), Yellowish Green PLBs (YG), Whitish Green PLBs (WG); Values represent mean ± SE of each experiment consist of five replicates.

Table 4: Mean increase in length (cm) and number of roots per seed derived seedlings of *D. aggregatum* in auxin supplemented full strength MS rooting media.

Sl. No.	PGR Concentration (mg/l)			Initial length (cm) per seed seedling (Mean ± SE)	Initial number of roots per seed derived seedling (Mean ± SE)	Increased length (cm) per seed derived seedling within 30d of culture (Mean ± SE)	Increased number of roots per seed derived seedling within 30d of culture (Mean ± SE)
	IAA	IBA	NAA				
1.	0.5	-	-	1.35±0.03	0.67±0.22	2.57±0.04	4.83±0.42
2.	1.0	-	-	1.30±0.01	0.50±0.24	2.60±0.05	4.33±0.48
3.	1.5	-	-	1.34±0.02	0.63±0.21	2.75±0.03	4.52±0.45
4.	-	0.5	-	1.33±0.02	1.00±0.39	2.75±0.06	4.67±0.44

5.	-	1.0	-	1.31±0.03	0.67±0.37	2.56±0.04	4.00±0.67
6.	-	1.5	-	1.35±0.01	0.85±0.35	2.68±0.04	4.53±0.57
7.	-	-	0.5	1.33±0.02	1.00±0.36	2.37±0.03	4.50±0.51
8.	-	-	1.0	1.35±0.01	0.67±0.34	2.29±0.05	5.00±0.31
9.	-	-	1.5	1.31±0.03	0.78±0.28	2.15±0.03	4.86±0.41
10.	0.5	0.5	-	1.35±0.02	1.00±0.35	2.75±0.05	5.00±0.35
11.	1.0	1.0	-	1.31±0.03	0.50±0.32	3.09±0.06	5.08±0.21
12.	1.5	1.5	-	1.33±0.02	0.75±0.27	2.92±0.06	4.73±0.25
13.	-	0.5	0.5	1.34±0.02	0.50±0.20	2.79±0.04	4.67±0.31
14.	-	1.0	1.0	1.33±0.04	1.00±0.38	3.43±0.05	5.25±0.37
15.	-	1.5	1.5	1.30±0.03	0.69±0.25	3.76±0.05	5.04±0.36
16.	0.5	-	0.5	1.31±0.02	0.83±0.39	2.82±0.03	4.83±0.45
17.	1.0	-	1.0	1.29±0.03	1.00±0.33	2.69±0.06	3.33±0.22
18.	1.5	-	1.5	1.34±0.02	0.73±0.25	2.84±0.05	3.76±0.22
19.	MS0 (Control)			1.30±0.01	1.00±0.00	1.67±0.04	3.16±0.18

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

Fig 1: Seeds germination of *D. aggregatum* on MS + 0.5 mg/l BAP + 0.5 mg/l NAA.

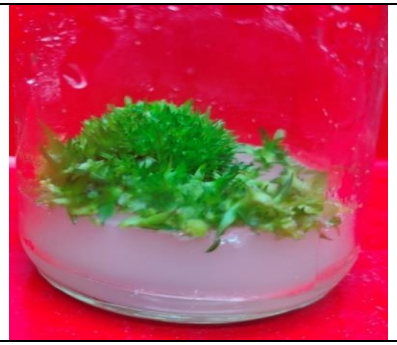

Fig. 2: Protocorm development of *D. aggregatum* on MS + 0.5 mg/l BAP + 0.5 mg/l NAA.

Fig 3: Development of plantlets of *D. aggregatum* on MS + 0.5 mg/l BAP + 0.5 mg/l NAA.

Fig 4: Elongation of germinated seedlings of *D. aggregatum* on MS + 1.5 mg/l BAP + 0.9 mg/l IAA.

Fig. 5: Development of MSBs in *D. aggregatum* developed from *in vitro* raised nodal segment on MS + 2.0 mg/l BAP + 1.0 mg/l NAA.

Fig 6: Development of PLBs in *D. aggregatum* sprouted from *in vitro* derived leaf segment on MS + 2.0 mg/l BAP + 1.0 mg/l IAA.

Fig 7: Development of strong and stout root system in seed derived seedlings of *D. aggregatum* on MS + 1.0 mg/l IBA + 1.0 mg/l NAA.

Fig 8: Transplantation of *D. aggregatum* in the outside environment.

Discussion

The severe structural limitations of *Dendrobium aggregatum* seeds particularly the profound lack of endosperm and a disorganized embryo prevent high frequency natural germination, making specialized *in vitro* techniques an absolute necessity for conservation (Arditti 1992, Rasmussen and Rasmussen 2014) [4, 14]. In the current study, the morphogenic superiority of the Murashige and Skoog (MS) basal medium over Knudson C (KC), Phytamax (PM) and Modified Vacin and Went (MVW) was distinctly evident for *D. aggregatum*. The superlative performance of fullstrength MS medium (yielding 100% germination) is principally attributed to its dense concentration of ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3), which supply the crucial macro nitrogen pools required for intensive protein synthesis and rapid embryonic cell division (Bhowmik and Rahman 2020, Jain 1997) [6, 14]. Similar dominance of MS medium in overriding the physiological barriers of recalcitrant orchid seeds has been corroborated in previous studies involving *Dendrobium chrysotoxum* (Kaur 2017) [15] and *Dendrobium primulinum* (Pant and Thapa 2012) [25], validating that high salt media significantly outpace the slower kinetics of MVW and KC formulations.

Beyond basal nutrients, the interplay of exogenous PGRs was the defining catalyst for morphogenic progression. The synergistic incorporation of 0.5 mg/l BAP and 0.5 mg/l NAA optimized both the germination percentage and the developmental timeline. Cytokinins (such as BAP) primarily orchestrate mitotic division in the undifferentiated embryonic mass, while auxins (such as NAA) are responsible for establishing axial polarity, triggering the structural transition from a swollen embryo into a functional protocorm (Arditti and Ernst 1993, Targu *et al*. 2023) [2, 32]. This harmonized PGRs ratio actively suppressed latency, pushing early leaf and root primordia differentiation months ahead of the PGRs free controls.

During the elongation phase, switching the cytokinin source from BAP to Kinetin (Kn) proved transformative. The peak longitudinal extension (2.88 cm increase) recorded on MS + 1.5 mg/l Kn + 0.9 mg/l IAA reflects Kinetin's specialized role in driving internodal cell wall plasticity and cellular expansion. While BAP is potent for bud proliferation, Kinetin coupled with IAA synergistically directs metabolic energy away from localized axillary node bursting and toward strictly vertical somatic elongation. This mechanism strongly aligns with findings in other epiphytic orchids, where specific Kn-IAA synergies mitigated vitrification and promoted robust vertical stem architecture (Mamun *et al.* 2018, Pant and Thapa 2012) [18, 25].

For mass commercial multiplication, manipulating explant topology through varied PGRs gradients yielded two distinct morphogenic pathways. Nodal segments directly formed multiple shoot buds (MSBs) *via* direct organogenesis, achieving a peak 72.47% response with 4.8 MSBs per explant under 2.0 mg/l BAP and 1.0 mg/l NAA. BAP fundamentally disrupts apical dominance, aggressively breaking the dormancy of the axillary meristems embedded within the node, while a moderate concentration of NAA ensures structural support and vascular connectivity for the newly emerging shoots (Martin and Madassery 2006, Reddy *et al.* 2021) [19, 29]. Alternatively, leaf segments underwent indirect embryogenesis, producing protocorm-like bodies (PLBs) at a 50% induction rate utilizing 2.0 mg/l BAP and 1.0 mg/l IAA. Epidermal and mesophyll cells in orchid leaves maintain a high degree of totipotency; the specific introduction of IAA (a naturally occurring, gentler auxin compared to synthetic NAA) in conjunction with high BAP triggers a cellular reprogramming event, dedifferentiating the tissue back into an embryonic state to form PLBs (Naing *et al.* 2011) [21]. The production of PLBs is highly advantageous for long term genetic preservation and automated bioreactor scaling (Bhowmik and Rahman 2020, Chugh *et al.* 2009) [6, 9].

The successful *ex vitro* transfer of micro-propagated orchids is entirely contingent upon the *in vitro* development of a prolific, highly functional rhizome network capable of rapid nutrient assimilation upon exposure to non-sterile substrates (Kauth *et al.* 2006, Pujari and Babu 2022) [16, 27]. Our data unequivocally demonstrate that dual-auxin combinations drastically outperform solitary treatments. The apex rhizogenesis formulation 1.0 mg/l IBA combined with 1.0 mg/l NAA generated thick, deeply ramified root systems (5.25 roots/shoot). Physiologically, IBA functions as a stable, slow release auxin precursor that provides a prolonged, non-toxic morphogenic signal, stimulating the continuous initiation of lateral root primordia. Simultaneously, NAA drives the rapid cell elongation necessary to push these primordia through the epidermis (Aktar *et al.* 2007, Paudel and Pant 2012) [1, 26]. The resulting dense root architecture directly contributed to the exceptional 80% survival rate during the critical acclimatization phase, where the porous coir/charcoal/brick matrix mimicked the natural epiphytic micro-canopy, ensuring superior aeration and preventing lethal fungal damping-off.

Conclusion

This study successfully establishes a highly efficient, end-to-end *in vitro* seed germination and micropropagation protocol for the medicinal orchid, *Dendrobium aggregatum* Roxb. The data confirm that full strength MS medium supplemented with precise synergistic ratios of auxins and cytokinins (optimally BAP and NAA for germination/multiplication and IBA with NAA for rooting) entirely bypasses the species' natural reproductive barriers. By enabling rapid direct organogenesis and robust PLBs formation, this protocol guarantees the continuous, mass scale production of genetically uniform, disease free clones. Consequently, this methodology not only serves as a vital framework for the commercial floricultural and pharmaceutical industries but also provides a scalable blueprint for the *ex situ* conservation and ecological reintroduction of *D. aggregatum* to the Chittagong Hill Tracts, Bangladesh.

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