International Journal of Biotechnology and Microbiology
www.biotechnologyjournals.com
Online ISSN: 2664-7680
Received: 27-06-2026, Accepted: 28-07-2026, Published: 12-08-2026
Volume 6, Issue 3, 2026, Page No. 42-50

In vitro seed germination and micropropagation of *Acampe rigida* (Buch.-Ham. ex Sm.) P.F.Hunt. - An indigenous medicinal orchid of Bangladesh

Md. Mustakim Zulfikar Alam, Md. Mahbubur Rahman, Tapash Kumar Bhowmik*
Plant Tissue Culture and Biotechnology Laboratory, Department of Botany, Faculty of Biological Sciences, University of Chittagong, Chattogram, Bangladesh
Corresponding Author: Tapash Kumar Bhowmik
DOI: https://doi.org/10.66856/ijbm.2026.8.3.8054

Abstract
Acampe rigida (Buch.-Ham. ex Sm.) P.F.Hunt. is an indigenous, epiphytic medicinal orchid of Bangladesh, highly valued for its therapeutic applications in treating joint pain, muscle stiffness and traumatic fractures. Due to extreme habitat destruction and the biological constraints of natural seed germination stemming from the absence of endosperm, this species faces severe conservation threats. The present study was undertaken to establish a highly efficient, reproducible *in vitro* propagation protocol exclusively focusing on asymbiotic seed germination and micropropagation *via* direct organogenesis and protocorm-like bodies (PLBs). Four basal media Murashige and Skoog (MS), Phytamax (PM), Modified Vacin and Went (MVW) and Knudson C (KC) were evaluated at varying strengths with and without plant growth regulators (PGRs). The maximum seed germination response (73.34%), the swiftest protocorm development (13.03 weeks) and the earliest differentiation of the 1st leaf primordia (17.07 ± 0.32 weeks) were achieved on full strength PM medium supplemented with 0.5 mg/l Benzyl Aminopurine (BAP) and 0.5 mg/l α-Naphthalene Acetic Acid (NAA). For rapid elongation of seed derived plantlets, MS medium fortified with 1.5 mg/l BAP and 0.9 mg/l NAA proved optimal, yielding an average length increase of 2.74 cm. High-frequency micropropagation was established using nodal and leaf explants from *in vitro* raised seedlings. Nodal segments exhibited the highest direct multiple shoot bud (MSB) induction (93.33% response; 6.2 MSBs/segment) on MS medium with 2.0 mg/l BAP and 1.0 mg/l NAA. Conversely, leaf segments underwent indirect embryogenesis, producing the maximum percentage of PLBs (77.33%) on the same medium matrix. Robust rhizogenesis was successfully induced on MS medium containing 0.5 mg/l Indole-3-Acetic Acid (IAA) and 0.5 mg/l NAA, generating the highest mean root length (4.03 cm) and root count (4.67 roots/seedling). Plantlets were successfully acclimatized ex *vitro* with a 75% survival rate. This standardized protocol provides a commercially viable pathway for the mass scale propagation and *ex situ* conservation of this endangered medicinal orchid.

Keywords: *Acampe rigida*, asymbiotic seed germination, conservation, direct organogenesis, micropropagation, protocorm-like bodies (PLBs)

Introduction

The Orchidaceae family represents the largest and most ecologically diverse group of flowering plants, comprising over 35,000 species distributed across 800 genera globally (Arditti 1979; Singh 2001) [2, 27]. Orchids have fascinated researchers not only for their immense floricultural appeal but also for their profound ethno-botanical and medicinal utility (Griesbach 2002) [11]. Historically, therapeutic applications of orchids have been extensively documented since the Vedic period in India and the early pharmacopoeias of China (Okhale *et al*. 2014, Toh 1994) [14, 28]. Among the ecologically rich zones of South Asia, the Chittagong Hill Tracts (CHT) of Bangladesh serve as a critical repository for diverse indigenous epiphytic orchids (Huda *et al*. 2006, Rahman *et al*. 2017) [14, 24].

Acampe rigida (Buch.-Ham. ex Sm.) P.F.Hunt., locally distributed in these hilly terrains, is a stout, epiphytic monopodial orchid characterized by fleshy, coriaceous leaves and dense, sub-corymbose inflorescences bearing fragrant, yellow-crimson spotted flowers. Beyond its ornamental value, *A. rigida* possesses substantial ethno-pharmacological importance. Its roots and leaves are traditionally utilized to relieve muscle tension, stimulate blood circulation, mitigate joint pain and heal traumatic fractures (Chuakul 2002, Huda and Kashem 2021) [7, 13]. However, indiscriminate wild harvesting for local medicine and illegal trade, coupled with rapid deforestation, has severely decimated the natural populations of this species, pushing it toward localized extinction.

The natural regeneration of *A. rigida*, like most orchids, is severely bottlenecked by its unique reproductive biology. Orchid seeds are microscopic, lack endosperm and possess minimal nutritional reserves (Arditti and Ernst 1993, Yam and Weatherhead 1991) [1, 30]. Consequently, *in vivo* germination necessitates a highly specific, obligate symbiotic association with mycorrhizal fungi to supply essential carbohydrates and nutrients during the early heterotrophic stages of embryo development (Hossain 2008, Rasmussen 1995) [12, 25]. This biological constraint results in less than 1% of seeds successfully germinating in nature. Therefore, conventional propagation methods are highly inefficient for commercial cultivation or large scale conservation efforts.

Plant tissue culture, specifically asymbiotic *in vitro* seed germination and clonal micropropagation, offers a robust

and scientifically validated alternative to circumvent these natural reproductive barriers (Chugh *et al*. 2009, Kauth *et al*. 2006) [8, 16]. While efficient *in vitro* propagation protocols have been widely standardized for commercial genera such as *Phalaenopsis*, *Dendrobium* and *Cymbidium* (Martin and Madassery 2006, Nayak *et al*. 1997) [18, 21], specific, high efficiency morphogenic pathways for indigenous medicinal species like *A. rigida* remain under explored. Micropropagation *via* direct organogenesis (multiple shoot buds, MSBs) or indirect somatic embryogenesis (protocorm-like bodies, PLBs) allows for the rapid, exponential generation of genetically uniform and disease free plantlets (Naing *et al*. 2011, Parab and Krishnan 2012) [20, 23]. The morphogenic responses of explants *in vitro* are heavily governed by the synergistic interaction of basal media composition and the precise ratios of exogenous plant growth regulators (PGRs), primarily auxins and cytokinins (Jain 1997, Seeni and Latha 2000) [15, 26].

Given the urgent need for *ex situ* conservation and the potential for commercial pharmaceutical exploitation, the present study was meticulously designed to standardize a reliable, rapid and mass scale *in vitro* propagation protocol for *A. rigida*. The explicit objectives were to: (i) evaluate the efficacy of four distinct basal media (MS, PM, MVW and KC) and varying PGR supplements on asymbiotic seed germination kinetics; (ii) optimize the cytokinin-auxin matrix for shoot elongation; (iii) establish high frequency micropropagation pathways utilizing nodal and leaf explants; and (iv) standardize a robust rooting and *ex vitro* acclimatization procedure for field establishment.

Materials and Methods

1. **Source of Plant Material and Explants:** Undehisced, mature green capsules of *Acampe rigida* were collected from the natural forest canopies of the Chittagong Hill Tracts, Bangladesh. For micropropagation phases, aseptically germinated, *in vitro* raised seedlings (5-6 cm in height) served as the primary source of sterile nodal and leaf explants.

2. **Surface Sterilization Protocol:** The collected capsules were thoroughly washed under running tap water to remove surface debris. They were subsequently agitated in a mild detergent solution containing 2-4 drops of Tween-20 for 15 minutes, followed by exhaustive rinsing with distilled water. Under the aseptic conditions of a laminar airflow cabinet, the capsules were surface sterilized using a 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10 minutes. This was followed by a 30-second immersion in 70% (v/v) ethanol. Finally, the capsules were rinsed 3-4 times with sterile double-distilled water to eliminate any residual sterilants and blotted dry on sterile Whatman filter paper.

3. **Basal Media Preparation and Culture Conditions:** Four distinct basal media formulations were evaluated for seed germination: Murashige and Skoog: MS (Murashige and Skoog 1962) [19], Phytamax: PM (Arditti 1977) [3], Modified Vacin and Went: MVW (Vacin and Went 1949) [29] and Knudson C: KC (Knudson 1946) [17]. The MS medium was supplemented with 3% (w/v) sucrose, while PM, MVW and KC were supplemented with 2% (w/v) sucrose. The pH of the MS medium was digitally adjusted to 5.8, whereas PM, MVW and KC media were adjusted to 5.4 using 1N NaOH or 1N HCl prior to the addition of 0.8% (w/v) bacteriological agar. The media were dispensed into appropriate culture vessels and autoclaved at 121°C and 1.9 kg/cm² pressure for 20 minutes. All cultures were incubated in a highly controlled growth room maintained at 25 ± 2°C under a 14/10-hour (light/dark) photoperiod provided by cool white fluorescent tubes emitting 4000-5000 lux illumination.

4. ***In vitro* Seed Germination and Shoot Elongation:** Surface sterilized capsules were longitudinally dissected using a sterile surgical scalpel and the powdery seeds were evenly scooped and inoculated onto the respective basal media (full and half strengths) supplemented with or without PGRs (0.5 mg/l BAP + 0.5 mg/l NAA). Following germination, primary seedlings exhibiting 1-2 nascent leaves were aseptically isolated and subcultured onto 25 diverse PGR configurations of full strength MS medium, incorporating varying concentrations of BAP, Kinetin (Kn), NAA and Indole-3-Acetic Acid (IAA) to facilitate rapid shoot elongation.

5. **Micropropagation *via* Nodal and Leaf Explants:** For mass micropropagation, nodal segments (1.0-1.5 cm) and leaf segments (0.5-1.0 cm) were excised from the elongated *in vitro* seedlings. These explants were inoculated onto full strength MS solid medium fortified with distinct ratios of BAP (1.0-3.0 mg/l) in combination with NAA, IAA, or Picloram (0.5-1.5 mg/l) to induce direct multiple shoot buds (MSBs) or indirect protocorm-like bodies (PLBs). Subculturing was systematically performed at 25-30 day intervals.

6. ***In vitro* Rooting and *Ex vitro* Acclimatization** Well developed, elongated shoots (2-3 cm) were individually excised and transferred to rooting matrices consisting of MS medium supplemented with isolated or combined concentrations (0.5-1.5 mg/l) of IAA, IBA and NAA. Upon the development of a robust radicular system, the seedlingss underwent a phased acclimatization process. Culture vessels were partially opened in the growth room for 24 hours, followed by exposure to ambient ex *vitro* conditions for gradually increasing intervals. Finally, the plantlets were washed free of agar and transplanted into community pots containing a sterilized, moistened matrix of small bricks, activated charcoal, wooden coal, sawdust and coconut husk (1:1:1:1:1 ratio) treated with a 0.1% fungicide solution.

7. **Experimental Design and Statistical Analysis**: All experiments were laid out in a Completely Randomized Design (CRD). Each treatment for germination comprised 15 replicates, while elongation, micropropagation and rooting experiments utilized 5 to 6 replicates per treatment. Data regarding germination percentages, morphogenic timelines, shoot lengths, MSBs/ PLBs induction rates and root metrics were recorded chronologically. Results are expressed as Mean ± Standard Error (SE).

Results

1. ***In vitro* Seed Germination:** The asymbiotic seed germination of *A. rigida* was evaluated across 12 variant media configurations encompassing full and half strengths of KC, MS, PM and MVW media, both with and without specific PGRs (0.5 mg/l BAP + 0.5 mg/l NAA). While germination was observed across all tested media, the morphogenic timelines and success percentages varied significantly (Table 1).

   The maximum germination response (73.34%) was recorded on full strength PM medium fortified with PGRs (Fig. 1). This was closely followed by full strength MS medium with PGRs, which yielded a 66.67% germination rate. Conversely, half strength, PGRs free MVW medium registered the minimum germination viability (13.34%).

   Chronologically, the inclusion of PGRs markedly accelerated early ontogeny. On full strength PM medium supplemented with PGRs, the initiation of germination occurred most rapidly (9.43 ± 0.25 weeks). This specific media matrix also required the least temporal duration to advance through subsequent developmental milestones: protocorm formation (13.03 ± 0.30 weeks, Fig. 2), differentiation of the first leaf primordia (17.07 ± 0.32 weeks), emergence of the first root primordia (21.10 ± 0.26 weeks) and complete seedling development (24.33 ± 0.27 weeks, Fig. 3). In stark contrast, PGRs free half strength MVW medium required the maximum time (18.20 ± 0.30 weeks) merely for the initiation of germination, completely failing to progress to advanced protocorm or leaf stages.

2. **Shoot Elongation of Seed Derived Plantlets:** Sub-culturing nascent seedlings onto diverse cytokinin and auxin matrices revealed distinct longitudinal growth dynamics over a 30-day culture span (Table 2). The combination of BAP and NAA proved vastly superior to Kn and IAA combinations for shoot elongation. The maximal mean increase in seedling length (2.74 ± 0.03 cm) was achieved on MS medium supplemented with 1.5 mg/l BAP and 0.9 mg/l NAA (Fig. 4). A marginally lower, yet highly effective elongation rate (2.69 ± 0.04 cm) was observed in the MS matrix containing 2.0 mg/l BAP and 1.2 mg/l NAA. The control medium lacking any PGRs exhibited the most suppressed elongation growth (1.82 ± 0.02 cm).]

3. **Micropropagation *via* Nodal and Leaf Explants:** To facilitate rapid mass multiplication, nodal and leaf segments sourced from *in vitro* plantlets were evaluated for their morphogenic potential (Table 3). Nodal segments primarily exhibited direct organogenesis, differentiating into multiple shoot buds (MSBs) without an intervening callus phase. The optimum response for nodal explants (93.33 ± 1.72% proliferation) was achieved on MS medium fortified with 2.0 mg/l BAP and 1.0 mg/l NAA, generating an average of 6.2 ± 0.58 MSBs per segment in a remarkably short duration of 5.20 ± 0.14 weeks (Fig. 5).

   Alternatively, leaf segments displayed high competence for indirect somatic embryogenesis, proliferating into distinct Protocorm-Like Bodies (PLBs). The same PGRs synergy (MS + 2.0 mg/l BAP + 1.0 mg/l NAA) proved most efficacious for foliar explants, yielding the highest percentage of greenish PLBs induction (77.33 ± 1.96%) within 5.60 ± 0.12 weeks (Fig. 6). The substitution of NAA with Picloram (MS + 2.0 mg/l BAP + 1.0 mg/l Pic) also yielded substantial PLBs proliferation (69.33 ± 1.96%).

4. **Rooting of *In vitro* Raised Plantlets**: For the establishment of an independent, functional radicular system, elongated shoots were cultured onto MS media containing solitary or combined auxins (Table 4). The co-application of IAA and NAA demonstrated clear synergistic superiority over IBA combinations. The maximum root proliferation parameters mean a longitudinal increase of 4.03 ± 0.03 cm and an average of 4.67 ± 0.21 robust roots per plantlet were documented on MS medium supplemented with 0.5 mg/l IAA and 0.5 mg/l NAA within 30 days of culture (Fig. 7). A comparable, yet slightly inferior response (3.95 ± 0.10 cm length; 4.50 ± 0.22 roots) was elicited using 0.5 mg/l IBA + 0.5 mg/l NAA. The PGRs free MS0 control medium generated the weakest rooting response (1.86 ± 0.03 cm length; 2.17 ± 0.17 roots).

5. **Acclimatization and Transplantation:** Following the establishment of a stout root system, the *in vitro* raised seedlings were successfully hardened using a phased exposure protocol. Transplanted to *ex vitro* community pots housing a porous, well aerated matrix (coir, sawdust, charcoal and brick pieces), the seedlings effectively resumed vegetative growth. A highly encouraging survival rate of 75% was recorded post acclimatization in the external greenhouse environment (Fig. 8).

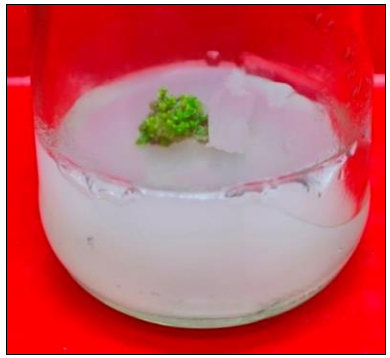

Fig 1: Seeds germination of A. rigida on PM + 0.5 mg/l BAP + 0.5 mg/l NAA.

Fig 2: Protocorm development of A. rigida on PM + 0.5 mg/l BAP + 0.5 mg/l NAA.

Fig 3: Development of plantlets of A. rigida on PM + 0.5 mg/l BAP + 0.5 mg/l NAA.

Fig 4: Elongation of germinated seedlings of *A. rigida* on MS + 1.5 mg/l BAP + 0.9 mg/l NAA.

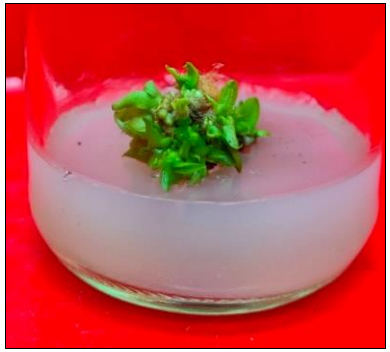

Fig 5: Development of MSBs in *A. rigida* developed from *in vitro* raised nodal segment on MS + 2.0

Fig 6: Development of PLBs in *A. rigida* sprouted from *in vitro* derived leaf segment on MS + 2.0

Fig 7: Development of strong and stout root system in seed derived seedlings of *A. rigida* on MS + 0.5

Fig 8: Transplantation of *A. rigida* in the outside environment.

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 Acampe rigida

Medium	Strength of medium	Time taken in weeks					% of culture vessel erminated	Remarks
		Initiation of germination (Mean ± SE)	Development of protocorms (Mean ± SE)	Differentiation of 1st leaf primordia (Mean ± SE)	Differentiation of 1st root primordia (Mean ± SE)	Development of seedlings (Mean ± SE)		
KC	Half without PGRs	18.13 ± 0.34	21.10 ± 0.35	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	13.34	+
	Full without PGRs	15.07 ± 0.27	19.07 ± 0.30	24.40 ± 0.31	28.17 ± 0.35	33.13 ± 0.31	33.34	+
	Full with PGRs	12.40 ± 0.31	16.40 ± 0.26	20.10 ± 0.36	25.07 ± 0.34	30.20 ± 0.29	46.67	+
MS	Half without PGRs	16.20 ± 0.33	22.10 ± 0.25	27.13 ± 0.32	32.20 ± 0.33	37.17 ± 0.26	33.34	+

	Full without PGRs	12.07 ± 0.32	17.13 ± 0.28	22.20 ± 0.30	26.07 ± 0.32	31.10 ± 0.32	53.34	++
	Full with PGRs	10.20 ± 0.30	14.27 ± 0.33	19.03 ± 0.33	23.10 ± 0.29	28.23 ± 0.34	66.67	++
PM	Half without PGRs	15.13 ± 0.34	21.20 ± 0.29	26.10 ± 0.35	31.13 ± 0.31	35.27 ± 0.27	46.67	+
	Full without PGRs	11.30 ± 0.29	16.27 ± 0.27	21.27 ± 0.28	24.37 ± 0.26	29.13 ± 0.29	60.00	++
	Fullwith PGRs	9.43 ± 0.25	13.03 ± 0.30	17.07 ± 0.32	21.10 ± 0.26	24.33 ± 0.27	73.34	++
MVW	Half without PGRs	18.20 ± 0.30	22.07 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	20.00	+
	Full without PGRs	16.20 ± 0.32	20.20 ± 0.35	24.20 ± 0.29	29.60 ± 0.34	34.20 ± 0.30	26.67	+
	Full with PGRs	13.13 ± 0.26	17.07 ± 0.28	21.37 ± 0.24	26.37 ± 0.24	30.07 ± 0.28	33.34	+

PGRs (0.5mg/l BAP + 0.5mg/l NAA); + = Minimum germination (0% ≤ + ≤ 49%), ++ = Medium germination (50% ≤ ++ ≤ 74%), +++ = Maximum germination (75% ≤ +++ ≤ 100%). Values represent mean ± SE of each experiment consist of 15 replicates.

Table 2: Elongation of *in vitro* germinated seedlings of A. rigida 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 after germination (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 ± 0.03	3.53 ± 0.03	2.19 ± 0.03
2.	1.0	-	-	-	1.31 ± 0.02	3.55 ± 0.04	2.23 ± 0.04
3.	1.5	-	-	-	1.35 ± 0.02	3.83 ± 0.06	2.48 ± 0.04
4.	2.0	-	-	-	1.40 ± 0.02	3.73 ± 0.05	2.33 ± 0.04
5.	-	0.5	-	-	1.37 ± 0.03	3.39 ± 0.06	2.02 ± 0.05
6.	-	1.0	-	-	1.33 ± 0.02	3.40 ± 0.05	2.06 ± 0.04
7.	-	1.5	-	-	1.42 ± 0.02	3.54 ± 0.06	2.12 ± 0.04
8.	-	2.0	-	-	1.39 ± 0.02	3.48 ± 0.06	2.09 ± 0.04
9.	0.5	-	0.3	-	1.36 ± 0.03	3.76 ± 0.05	2.41 ± 0.04
10.	1.0	-	0.6	-	1.38 ± 0.02	4.02 ± 0.04	2.64 ± 0.04
11.	1.5	-	0.9	-	1.32 ± 0.03	4.05 ± 0.02	2.74 ± 0.03
12.	2.0	-	1.2	-	1.41 ± 0.02	4.10 ± 0.05	2.69 ± 0.04
13.	0.5	-	-	0.3	1.37 ± 0.03	3.67 ± 0.02	2.30 ± 0.04
14.	1.0	-	-	0.6	1.33 ± 0.02	3.76 ± 0.02	2.43 ± 0.03
15.	1.5	-	-	0.9	1.40 ± 0.02	4.07 ± 0.04	2.67 ± 0.04
16.	2.0	-	-	1.2	1.35 ± 0.02	3.94 ± 0.03	2.58 ± 0.03
17.	-	0.5	0.3	-	1.39 ± 0.03	3.65 ± 0.06	2.26 ± 0.04
18.	-	1.0	0.6	-	1.36 ± 0.03	3.74 ± 0.04	2.38 ± 0.03
19.	-	1.5	0.9	-	1.32 ± 0.03	3.94 ± 0.04	2.62 ± 0.04
20.	-	2.0	1.2	-	1.38 ± 0.02	3.89 ± 0.05	2.51 ± 0.04

21.	-	0.5	-	0.3	1.42 ± 0.02	3.58 ± 0.02	2.16 ± 0.03
22.	-	1.0	-	0.6	1.34 ± 0.02	3.69 ± 0.04	2.35 ± 0.04
23.	-	1.5	-	0.9	1.41 ± 0.02	3.97 ± 0.03	2.55 ± 0.03
24.	-	2.0	-	1.2	1.36 ± 0.03	3.82 ± 0.05	2.46 ± 0.03
25.	MS0 (Control)				1.39 ± 0.03	3.21 ± 0.03	1.82 ± 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 A. rigida 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	-	-	-	-	32.00 ± 1.44	7.26 ± 0.09	2.2 ± 0.49	0.00 ± 0.00	0.00 ± 0.00	-
2.	2.0	-	-	-	-	32.00 ± 1.44	7.12 ± 0.12	2.4 ± 0.24	6.67 ± 0.00	8.34 ± 0.08	G
3.	3.0	-	-	-	-	25.33 ± 2.24	7.36 ± 0.09	1.8 ± 0.37	18.67 ± 1.44	7.62 ± 0.11	YG
4.	-	1.0	-	-	-	18.67 ± 1.44	7.44 ± 0.11	1.6 ± 0.40	0.00 ± 0.00	0.00 ± 0.00	-
5.	-	2.0	-	-	-	25.33 ± 2.24	7.20 ± 0.14	2.0 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	-
6.	-	3.0	-	-	-	18.67 ± 1.44	7.42 ± 0.10	1.4 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	-
7.	1.0	-	0.5	-	-	88.00 ± 2.24	5.46 ± 0.09	5.8 ± 0.58	52.00 ± 1.44	6.48 ± 0.08	WG
8.	2.0	-	1.0	-	-	93.33 ± 1.72	5.20 ± 0.14	6.2 ± 0.58	77.33 ± 1.96	5.60 ± 0.12	G
9.	3.0	-	1.5	-	-	81.33 ± 2.24	5.64 ± 0.12	5.4 ± 0.51	64.00 ± 1.96	6.12 ± 0.12	YG
10.	1.0	-	-	0.5	-	76.00 ± 1.96	5.72 ± 0.14	5.2 ± 0.58	37.33 ± 1.96	7.08 ± 0.10	WG
11.	2.0	-	-	1.0	-	82.67 ± 2.61	5.58 ± 0.12	5.6 ± 0.51	56.00 ± 1.96	6.26 ± 0.09	G
12.	3.0	-	-	1.5	-	69.33 ± 1.96	6.12 ± 0.12	4.8 ± 0.37	50.67 ± 1.96	6.54 ± 0.08	G
13.	1.0	-	-	-	0.5	64.00 ± 1.96	6.26 ± 0.09	4.4 ± 0.51	44.00 ± 1.54	6.68 ± 0.16	YG
14.	2.0	-	-	-	1.0	76.00 ± 2.31	6.02 ± 0.15	5.0 ± 0.71	69.33 ± 1.96	6.02 ± 0.13	G
15.	3.0	-	-	-	1.5	58.67 ± 1.44	6.34 ± 0.11	4.0 ± 0.71	57.33 ± 1.96	6.34 ± 0.09	WG
16.	-	1.0	0.5	-	-	69.33 ± 1.96	6.12 ± 0.12	4.6 ± 0.51	20.00 ± 1.72	7.72 ± 0.14	G
17.	-	2.0	1.0	-	-	62.67 ± 2.61	6.34 ± 0.09	4.2 ± 0.37	44.00 ± 1.96	6.74 ± 0.15	G
18.	-	3.0	1.5	-	-	45.33 ± 1.44	6.72 ± 0.16	3.0 ± 0.71	32.00 ± 1.44	7.26 ± 0.09	YG
19.	-	1.0	-	0.5	-	56.00 ± 1.96	6.44 ± 0.11	3.8 ± 0.58	13.33 ± 1.72	8.12 ± 0.12	WG
20.	-	2.0	-	1.0	-	50.67 ± 1.96	6.52 ± 0.14	3.6 ± 0.40	32.00 ± 1.44	7.34 ± 0.11	G
21.	-	3.0	-	1.5	-	37.33 ± 1.96	7.04 ± 0.16	2.6 ± 0.40	25.33 ± 2.24	7.52 ± 0.15	YG
22.	-	1.0	-	-	0.5	45.33 ± 2.24	6.66 ± 0.16	3.2 ± 0.58	13.33 ± 1.22	8.08 ± 0.15	G
23.	-	2.0	-	-	1.0	52.00 ± 2.24	6.52 ± 0.14	3.4 ± 0.51	38.67 ± 1.44	7.10 ± 0.14	YG
24.	-	3.0	-	-	1.5	37.33 ± 1.96	7.02 ± 0.15	2.8 ± 0.58	25.33 ± 1.44	7.46 ± 0.09	G
25.	MS0 (Control)					13.33 ± 1.72	7.64 ± 0.12	1.0 ± 0.00	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 plantlets of A. rigida in auxin supplemented full strength MS rooting media.

Sl. No.	PGR Concentration (mg/l)			Initial length (cm) per seed derived 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	-	-	1.41 ± 0.02	1.00 ± 0.37	3.20 ± 0.07	3.67 ± 0.42
2.	1.0	-	-	1.40 ± 0.03	1.17 ± 0.31	2.94 ± 0.09	3.00 ± 0.52
3.	1.5	-	-	1.39 ± 0.03	0.83 ± 0.31	2.05 ± 0.07	3.67 ± 0.33
4.	-	0.5	-	1.43 ± 0.03	1.17 ± 0.31	3.32 ± 0.08	3.67 ± 0.42
5.	-	1.0	-	1.45 ± 0.03	0.67 ± 0.33	3.02 ± 0.08	3.17 ± 0.31
6.	-	1.5	-	1.37 ± 0.03	1.00 ± 0.26	2.18 ± 0.10	2.50 ± 0.34
7.	-	-	0.5	1.33 ± 0.01	0.83 ± 0.31	3.65 ± 0.07	4.00 ± 0.37
8.	-	-	1.0	1.42 ± 0.03	0.50 ± 0.22	3.11 ± 0.09	3.33 ± 0.42
9.	-	-	1.5	1.40 ± 0.03	1.00 ± 0.26	2.34 ± 0.05	2.67 ± 0.33
10.	0.5	0.5	-	1.36 ± 0.02	0.83 ± 0.31	3.72 ± 0.08	4.17 ± 0.31
11.	1.0	1.0	-	1.37 ± 0.02	1.17 ± 0.40	3.41 ± 0.08	3.83 ± 0.31
12.	1.5	1.5	-	1.34 ± 0.01	0.67 ± 0.33	2.94 ± 0.09	2.67 ± 0.33
13.	-	0.5	0.5	1.39 ± 0.02	0.83 ± 0.31	3.95 ± 0.10	4.50 ± 0.22
14.	-	1.0	1.0	1.35 ± 0.02	1.00 ± 0.37	3.54 ± 0.06	3.83 ± 0.40
15.	-	1.5	1.5	1.38 ± 0.02	1.17 ± 0.31	2.66 ± 0.07	2.83 ± 0.31
16.	0.5	-	0.5	1.46 ± 0.02	0.83 ± 0.31	4.03 ± 0.03	4.67 ± 0.21
17.	1.0	-	1.0	1.44 ± 0.02	0.67 ± 0.21	3.86 ± 0.07	4.33 ± 0.21
18.	1.5	-	1.5	1.39 ± 0.02	0.50 ± 0.22	2.78 ± 0.08	3.00 ± 0.26
19.	MS0 (Control)			1.37 ± 0.02	0.83 ± 0.17	1.86 ± 0.03	2.17 ± 0.17

Values represent mean ± SE of each experiment consist of six replicate

Discussion

The optimization of basal nutrient media and exogenous PGRs supplementation is the most critical determinant for circumventing the natural regenerative barriers of orchids (Arditti 1992, Murashige and Skoog 1962) [4, 19]. The present investigation delineates a highly reproducible micropropagation pathway for *Acampe rigida*, successfully bridging the gap between seed germination limitations and mass *ex situ* conservation requirements.

In evaluating asymbiotic seed germination, nutritional composition played an overriding role. While seeds successfully germinated across KC, MS, PM and MVW media, PM medium (Phytamax) fortified with PGRs proved unequivocally superior (73.34% germination in 9.43 weeks). This enhanced morphogenic response can be scientifically attributed to the rich organic nitrogen complexes inherent in the PM formulation, predominantly peptone, coupled with its highly concentrated vitamin profile. Peptone acts as a readily assimilable, highly mobile source of amino acids, which is profoundly vital during the early heterotrophic stage of orchid protocorm development before the acquisition of full photosynthetic competence (Arditti and Ernst 1993, Vacin and Went 1949) [1, 29]. In contrast, the KC medium often historically employed for terrestrial taxa proved too nutritionally sparse for *A. rigida*, resulting in the lowest germination metrics. The synergistic application of 0.5 mg/l BAP and 0.5 mg/l NAA was fundamental in breaking seed dormancy, validating the established biological paradigm that a balanced exogenous supply of cytokinins (for intense cell division) and auxins (for axis elongation) orchestrates the rapid structural transition from undifferentiated embryos to polarized protocorms (Chugh *et al*. 2009, Kauth *et al*. 2006) [8, 16].

During the shoot elongation phase, the continuous application of BAP alongside NAA significantly outperformed Kn and IAA combinations, yielding a maximal length increase of 2.74 cm. BAP is widely recognized as a highly potent, stable and less degradable aromatic cytokinin compared to Kinetin. It aggressively stimulates the shoot apical meristem and facilitates rapid inter-nodal elongation when counterbalanced with a stabilizing auxin like NAA (Naing *et al*. 2011, Parab and Krishnan 2012) [20, 23]. This corroborates similar physiological trends observed in the *in vitro* elongation of other medicinal epiphytes (Seeni and Latha 2000) [26].

Mass micropropagation was remarkably successful using both nodal and foliar explants, though *via* distinct organogenic pathways. Nodal segments, possessing pre-existing axillary meristems, bypassed callogenesis to directly yield multiple shoot buds (MSBs). The application of 2.0 mg/l BAP paired with 1.0 mg/l NAA triggered optimal axillary bud breaking (93.33% response), effectively disrupting apical dominance and redirecting cellular energy toward lateral meristematic proliferation (Jain 1997) [15]. Conversely, leaf explants exhibited a profound capacity for indirect somatic embryogenesis, differentiating into mass Protocorm-Like Bodies (PLBs). The induction of PLBs from leaf tissue requires extensive cellular dedifferentiation and genetic reprogramming, a highly energy intensive process expertly facilitated by the exact same BAP/NAA ratio (2.0 mg/l / 1.0 mg/l). The success of NAA over other auxins (like IAA) in maintaining morphogenic stability during PLBs induction lies in its synthetic nature, rendering it less susceptible to rapid enzymatic degradation by plant peroxidases, thus providing a sustained morphogenic stimulus (Bhadra and Hossain 2003, Martin and Madassery 2006) [5, 18].

A robust radicular network is the ultimate prerequisite for *ex vitro* survival. In the present study, rhizogenesis was heavily auxin dependent, with the synergistic interaction of 0.5 mg/l IAA and 0.5 mg/l NAA generating the most extensive root architecture (4.67 roots per shoot). While IBA is classically considered the premier rooting hormone due to its slow release characteristics (De *et al*. 2014) [9], the specific biochemical physiology of *A. rigida* responded better to the combined application of a natural auxin (IAA) for rapid root initiation and a synthetic auxin (NAA) for sustained root elongation and lateral root branching. This phenomenon of "Auxin Synergism" is well documented in the commercial propagation of monopodial orchids (Goh and Tan 1982) [10].

The ultimate acclimatization survival rate of 75% is highly encouraging for an epiphytic species transitioning from a hyper humid *in vitro* environment to fluctuating greenhouse conditions. The use of a highly porous potting matrix (charcoal, coir, sawdust) successfully mimicked the natural, well aerated epiphytic habitat, preventing root rot while retaining sufficient moisture to mitigate desiccation shock (Chowdhury 2001) [6].

Conclusion

The present investigation establishes a highly efficient, commercially viable and scientifically rigorous protocol for the mass-scale micropropagation and *in vitro* seed germination of the endangered medicinal orchid *Acampe rigida*. The study unequivocally demonstrates that Phytamax (PM) medium provides the optimal nutritional matrix for asymbiotic seed germination, while Murashige and Skoog (MS) medium fortified with a highly specific 2:1 ratio of BAP (2.0 mg/l) and NAA (1.0 mg/l) maximizes shoot multiplication through both direct organogenesis and PLBs pathways. Furthermore, a synergistic auxin treatment (IAA + NAA) ensures the development of a robust root system, leading to successful *ex vitro* acclimatization. This optimized protocol serves as a crucial biotechnological tool to mitigate wild harvesting pressures, aiding in the *ex situ* conservation of *A. rigida* and offers a sustainable platform for its commercial cultivation to supply the global pharmaceutical industry.

Acknowledgement

The authors extend their sincere gratitude to the Plant Tissue Culture and Biotechnology Laboratory, Department of Botany, Faculty of Biological Sciences, University of Chittagong, Bangladesh, for providing the essential laboratory infrastructure, technical resources and continuous support necessary for the successful execution of this research.

References

1. Arditti J, Ernst R. Micropropagation of orchids. New York: John Wiley & Sons, Inc., 1993, 467-520.

2. Arditti J. Aspects of the physiology of orchids. Advances in Botanical Research,1979:7:421-655.
3. Arditti J. Clonal propagation of orchids by means of tissue culture: A manual. In: Arditti J (ed.). Orchid Biology: Reviews and Perspectives I. Ithaca, New York: Cornell University Press, 1977, 203-293.
4. Arditti J. Fundamentals of Orchid Biology. New York: John Wiley & Sons, Inc., 1992.
5. Bhadra SK, Hossain MM. *In vitro* Germination and Micropropagation of *Geodorum densiflorum* (Lam.) Schltr., an Endangered Orchid Species. Plant Tissue Culture,2003:13(2):165-171.
6. Chowdhury HJ. Orchid Diversity in North East India. Sixth National Seminar on Orchid Diversity in India Science and Commerce and Orchid show,2001:15:1-17.
7. Chuakul W. Ethnomedical uses of Thai Orchidaceous plants. Mohidol Journal of Pharmaceutical Sciences,2002:29(3-4):41-45.
8. Chugh S, Guha S, Rao IU. Micropropagation of orchids: a review on the potential of different explants. Scientia Horticulturae,2009:122(4):507-520.
9. De LC, Vij SP, Medhi RP. Post-Harvest Physiology and Technology in Orchids. Journal of Horticulture,2014:1(1):102-102. doi:10.4172/horticulture.1000102.
10. Goh CJ, Tan H. Clonal propagation from leaf explants in *Renantanda* orchid hybrid. Orchid Review,1982:90:295-296.
11. Griesbach RJ. Development of *Phalaenopsis* orchids for the mass-market. In: Janick J, Whipkey A (eds.), Trends in new crops and new uses. Alexandria, VA: ASHS Press, 2002, 458-465.
12. Hossain MM. Symbiotic seed germination and *in vitro* seedling development of *Epidendrum ibaguense* Kunth. African Journal of Biotechnology,2008:7(20):3614-3619.
13. Huda MK, Kashem MA. Phytochemistry of medicinal orchids, Bangladesh perspective. Chattaogram, Bangladesh: Research and Publication Cell, University of Chittagong, 2021.
14. Huda MK, Wilcock CC, Rahman MA. The Ethnobotanical Information on Indigenous Orchids of Bangladesh. Hamdard Medicus,2006:49(3):138-143.
15. Jain RK. Effects of some factor on plant regeneration from *indica* rice cells and protoplasts: A review. Indian Journal of Biology,1997:35:323-331.
16. Kauth PJ, Vendrame WA, Kane ME. Micropropagation of *Calopogon tuberosus*, a native terrestrial orchid, by shoot production from corm explants. *In vitro* Cellular & Developmental Biology - Plant,2006:42:23-30.
17. Knudson L. A nutrient for germination of orchid seeds. American Orchid Society Bulletin,1946:15:214-217.
18. Martin KP, Madassery J. Rapid *in vitro* propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants and protocorm-like bodies. Scientia Horticulturae,2006:108(1):95-99. doi:10.1016/j.scienta.2006.01.011.
19. Murashige T, Skoog F. A revised medium for rapid growth and bio-assay with tobacco tissue cultures. Physiologia Plantarum,1962:15:473-497.
20. Naing AH, Chung JD, Lim KB. Plant regeneration through indirect somatic embryogenesis in *Coelogyne cristata* orchid. American Journal of Plant Sciences,2011:2:262-267.
21. Nayak NR, Rath SP, Patnaik S. *In vitro* propagation of three epiphytic orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch.-Ham.) Sw. through thidiazuron-induced high-frequency shoot proliferation. Scientia Horticulturae,1997:71(3-4):243-250.
22. Okhale SE, Ugbabe GE, Bamidele O, Ajoku GA, Eghareba HO. Phytochemical and antimicrobial studies on extractions of *Calaptrochilum emarginatum* (Sw) Schltr (*Orchidaceae*) growing in Nigeria. Journal of Medicinal Plants Research,2014:8(4):223-228.
23. Parab GV, Krishnan S. Rapid *in vitro* Mass Multiplication of Orchids *Aerides maculosa* Lindl. and *Rhynchostylis retusa* (L.) Bl. from Immature Seeds. Indian Journal of Biotechnology,2012:11:288-294.
24. Rahman MA, Huda MK, Rashid ME. Orchid Species Diversity in Bangladesh and their Revised Nomenclatural Updates. Biodiversity Bulletin Bangladesh,2017:10:1-70.
25. Rasmussen HN. Terrestrial Orchids: From Seed to Mycotrophic Plant. Cambridge: Cambridge University Press, 1995.
26. Seeni S, Latha PG. *In vitro* multiplication and ecorehabilitation of the endangered Blue *Vanda*. Plant Cell, Tissue and Organ Culture,2000:61(1):1-8. doi:10.1023/A:1006444614657.
27. Singh K. Commercial cut-flower production of tropical orchids. In: proceedings of sixth National Seminar on Orchids Diversity of India: Science and Commerce and Orchid Show, 2001, 39-44.
28. Toh CA. Pharmacognostic studies in *Orchidaceae* plants. Korean Journal of Pharmacognosy,1994:25:293-304.
29. Vacin E, Went FW. Some pH changes in nutrient solution. Botanical Gazette,1949:110(4):605-613.
30. Yam TW, Weatherhead MA. Leaf-tip culture of several native orchids of Hong Kong. Lindleyana,1991:6(3):147-150.