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In vitro seed germination, differentiation and PLBs multiplication of the medicinal orchid *Pholidota imbricata* Hook. F. from Bangladesh

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Abstract
Pholidota imbricata Hook. f. is an epiphytic medicinal orchid native to Bangladesh, valuable for its therapeutic applications in treating rheumatic pain and bone fractures. Due to habitat destruction and the inherent difficulty of natural seed germination caused by the lack of endosperm, this species faces conservation threats. This study established a reliable *in vitro* propagation protocol *via* asymbiotic seed germination and Protocorm-Like Bodies (PLBs) multiplication. Four basal media *viz*. Murashige and Skoog (MS), Phytamax (PM), Modified Vacin and Went (MVW) and Knudson C (KC) were evaluated. The highest seed germination rate (93.34%) and earliest protocorm development (8.13 weeks) were achieved on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. For mass multiplication, PLBs were cultured on MS medium fortified with various concentrations of 2,4-D and BAP. The synergistic combination of 2.0 mg/l 2,4-D and 0.8 mg/l BAP proved most effective, yielding the highest fresh weight (1.53 g) and number of PLBs (88.2) per vessel after 60 days of inoculation. Regenerated plantlets were successfully acclimatized with a 65% survival rate. This protocol provides a viable pathway for the mass propagation and *ex situ* conservation of this important medicinal orchid.

Keywords: *Pholidota imbricata*, asymbiotic seed germination, protocorn-like bodies, *ex situ* conservation, acclimatization

Introduction
The Orchidaceae family, one of the largest and most diverse groups of flowering plants, exhibits complex reproductive strategies. Despite producing millions of seeds per capsule, natural regeneration is severely limited because orchid seeds lack endosperm and require specific mycorrhizal fungal associations to germinate [1]. *Pholidota imbricata* Hook. f., locally known as the "Necklace Orchid," is distributed across the Chittagong Hill Tracts and Sylhet regions of Bangladesh. It holds significant ethnomedicinal value; its pseudobulbs and roots are traditionally used to treat rheumatic pain, abdominal discomfort and bone fractures [2-3].

However, the population of *P. imbricata* is declining due to indiscriminate collection, deforestation and slow natural growth rates. Conventional propagation methods are often insufficient to meet commercial and conservation demands. Plant tissue culture, specifically asymbiotic seed germination, offers a powerful tool for overcoming dormancy and producing mass planting material [4]. While protocols exist for various commercial orchids like *Dendrobium* and *Phalaenopsis*, specific high efficiency protocols for indigenous medicinal orchids of Bangladesh remain under researched.

This study aims to standardize an efficient *in vitro* protocol for *P. imbricata* by evaluating the efficacy of different basal media (MS, PM, MVW, KC) on germination and optimizing plant growth regulators (PGRs) for rapid PLB multiplication and differentiation.

Materials and Methods
Plant Material and Sterilization
Green capsules of *Pholidota imbricata* were collected from Naikhongchhari, Bandarban (Chittagong Hill Tracts). The capsules were washed under running tap water, treated with a detergent solution and surface sterilized with 0.2% (w/v) mercuric chloride ($HgCl_2$) for 5 minutes. This was followed by a one-minute rinse in 70% ethanol and three-four times washes with sterile double distilled water.

Media Preparation
Four basal media with or without PGRs were prepared to test germination efficiency:

1. **MS:** Murashige and Skoog (1962) [5] + 3% sucrose.
2. **PM:** Phytamax (Arditti, 1977) [6] + 2% sucrose.
3. **MVW:** Modified Vacin and Went (1949) [7] + 2% sucrose.
4. **KC:** Knudson C (1946) [8] + 2% sucrose.

All media were gelled with 0.8% agar (Fluka) and the pH was adjusted (5.8 for MS; 5.4 for PM, MVW; 5.0 for KC) prior to autoclaving at 121°C for 20 minutes.

Seed Germination and Differentiation
Seeds were scooped from sterilized capsules and inoculated onto the four basal media, both with and without Plant Growth Regulators (PGRs). The primary PGR combination tested for germination enhancement was 0.5 mg/l Benzyl Adenine (BAP) + 0.5 mg/l Naphthalene Acetic Acid (NAA). Cultures were incubated at 25 ± 2 °C, 2500-3000 lux Light intensity under a 14/10-hour light/dark photoperiod.

PLBs Multiplication
Protocorm-Like Bodies (PLBs) derived from germinated seeds were subcultured onto MS medium supplemented with 2, 4-Dichlorophenoxyacetic acid (2,4-D) alone or in combination with BAP or Kinetin (Kn) to induce mass multiplication. Data on fresh weight and PLBs number were recorded at 30 and 60 days after inoculation (DAI).

Hardening
Well-developed seedlings with stout roots were sequentially acclimatized. Culture vessels were opened for 24 hours,

then plantlets were removed, washed and planted in pots containing a mixture of moistened coir, sawdust and coal.

Statistical Analysis

Data were recorded on germination percentage, time taken for developmental stages, PLBs weight and number. Results are presented as Mean ± Standard Error (SE).

Results

The seeds of medicinal orchid *Pholidota* i*mbricata* were aseptically grown on four media namely MS [5], PM [6], MVW [7] and KC [8] medium supplemented with or without PGRs (Table 1, Figures 1-2). The seeds of this species germinated on all of the media with or without PGRs but the germination percentage of cultured vessel were varied in different media conditions. The highest percentage of germination on culture vessel was noted on PGRs (0.5 mg/l BAP + 0.5 mg/l NAA) fortified MS medium (93.34%) followed by PGRs supplemented PM medium (86.67%). The poor germination rate was observed on PGRs free KC medium (46.67%). The initiation of germination of seeds was recorded within the lowest time (6.20 ± 0.35 weeks) on PGRs fortified MS medium; whereas, the maximum time (12.43 ± 0.35 weeks) was required on KC basal medium. PGRs supplemented MS medium was best and required less time for the development of protocorms (8.13 ± 0.36 weeks), differentiation of first leaf primordia (12.30 ± 0.37 weeks), differentiation of first root primordia (17.40 ± 0.36 weeks), development of seedlings (23.10 ± 0.35 weeks) followed by PGRs fortified PM medium (10.37 ± 0.45, 15.47 ± 0.39, 21.37 ± 0.39 and 26.17 ± 0.42 weeks) respectively. The poor responses was recorded on KC basal medium and needed highest time for the development of protocorms (15.43 ± 0.39 weeks), differentiation of first leaf primordia (21.40 ± 0.41 weeks), differentiation of first root primordia (28.23 ± 0.35 weeks), development of seedlings (35.37 ± 0.34 weeks). So it can be acclaimed that MS medium with addition of PGRs showed best result of germination and also took less time to germinate than the other media subsequently PM, MVW and KC.

In vitro raised PLBs were cultured on 2,4-D individually and in combination with BAP or Kn supplemented agar solidified MS medium for increasing the weight and number of PLBs. These PLBs were cultured independently on PGRs supplemented medium for the regeneration of plantlets quickly and within a short time produce a huge number of plantlets from the cultured PLBs. PLBs multiplication was superior in 2,4-D and BAP supplemented MS medium than Kn.

The topmost increase of PLBs (0.62 ± 0.02 g, 32.8 ± 0.58 no after 30d; 1.53 ± 0.03 g, 88.2 ± 0.86 no after 60d) multiplication of *P. imbricata* was recorded on MS medium fortified with 2.0 mg/l 2,4-D and 0.8 mg/l BAP (Table 2, Figures 3-4). The MS medium containing 1.5 mg/l 2,4-D and 0.6 mg/l BAP gave almost similar results for multiplication of PLBs (0.59 ± 0.03 g, 29.6 ± 0.93 no after 30d; 1.47 ± 0.02 g, 83.6 ± 0.93 no after 60d). The induced PLBs were greenish in colour and friable in texture. The lowest PLBs multiplication after 30d (0.45 ± 0.02 g, 16.6 ± 0.93 no) and after 60d (0.86 ± 0.03 g, 31.4 ± 0.81 no) of culture was obtained in MS medium supplemented with 0.5 mg/l 2,4-D and 0.2 mg/l Kn.

Seedlings transferred to the potting mixture (coir, sawdust, coal) showed a survival rate of 75%. The surviving plants established new root systems and resumed growth within 3 weeks.

Table 1: Effect of KC, MS, PM and MVW media with or without PGRs on *in vitro* seed germination, differentiation and seedlings development of *P.* imbricate

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 leaf primordia (Mean ± SE)	Differentiation of 1st root primordia (Mean ± SE)	Development of seedlings (Mean ± SE)		
KC	Full without PGRs	12.43 ± 0.35	15.43 ± 0.39	21.40 ± 0.41	28.23 ± 0.35	35.37 ± 0.34	46.67	+
	Full with PGRs	10.30 ± 0.41	12.23 ± 0.35	17.13 ± 0.33	23.17 ± 0.36	30.47 ± 0.35	66.67	++
MS	Full without PGRs	9.13 ± 0.33	12.47 ± 0.34	17.17 ± 0.34	23.47 ± 0.37	30.43 ± 0.34	80.00	+++
	Full with PGRs	6.20 ± 0.35	8.13 ± 0.36	12.30 ± 0.37	17.40 ± 0.36	23.10 ± 0.35	93.34	+++
PM	Full without PGRs	10.40 ± 0.39	13.43 ± 0.35	19.37 ± 0.39	26.50 ± 0.34	31.13 ± 0.36	73.34	+++
	Full with PGRs	7.17 ± 0.40	10.37 ± 0.45	15.47 ± 0.39	21.37 ± 0.39	26.17 ± 0.42	86.67	+++
MVW	Full without PGRs	11.17 ± 0.36	15.30 ± 0.33	20.17 ± 0.36	25.37 ± 0.38	33.40 ± 0.42	53.34	++
	Full with PGRs	9.47 ± 0.33b	11.30 ± 0.40	16.43 ± 0.32	20.43 ± 0.40	28.47 ± 0.37	73.34	++

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 12 replicates.

Table 2: Effect of 2, 4-D individually and in combination with BAP or KN in MS medium on PLBs multiplication of *P. imbricata*.

Sl. No.	PGRs Concentration (mg/l)			Increased weight of PLBs (g/vessel) (Mean ± SE)		Increased number of PLBs (No/vessel) (Mean ± SE)		Quantity of PLBs	Colour and texture of induced PLBs
	2,4-D	BAP	Kn	30 DAI	60 DAI	30 DAI	60 DAI		
1.	0.5	-	-	0.47±0.02	0.97±0.02	18.0±0.71	37.4±0.93	Few	GYF
2.	1.0	-	-	0.49±0.02	1.09±0.03	20.2±0.73	45.6±0.81	Few	GC
3.	1.5	-	-	0.52±0.03	1.26±0.03	23.2±0.58	58.2±0.73	Moderate	GYC
4.	2.0	-	-	0.56±0.02	1.42±0.02	27.4±0.87	74.0±0.71	Many	YGC
5.	2.5	-	-	0.57±0.03	1.44±0.03	28.0±0.71	79.6±0.75	Many	YGF
6.	0.5	0.2	-	0.51±0.02	1.22±0.03	22.6±0.93	55.4±0.68	Moderate	YGC
7.	1.0	0.4	-	0.55±0.02	1.38±0.03	26.6±0.51	71.0±0.71	Many	WGC
8.	1.5	0.6	-	0.59±0.03	1.47±0.02	29.6±0.93	83.6±0.93	Many	GYC
9.	2.0	0.8	-	0.62±0.02	1.53±0.03	32.8±0.58	88.2±0.86	Many	GF
10.	2.5	1.0	-	0.48±0.03	1.04±0.03	19.4±0.68	41.0±0.71	Few	YGF
11.	0.5	-	0.2	0.45±0.02	0.86±0.03	16.6±0.93	31.4±0.81	Few	WGC
12.	1.0	-	0.4	0.50±0.03	1.15±0.02	21.0±0.71	50.4±0.93	Moderate	WGC
13.	1.5	-	0.6	0.53±0.03	1.31±0.03	24.6±0.51	61.0±0.71	Moderate	GYC
14.	2.0	-	0.8	0.54±0.03	1.35±0.02	25.4±0.93	67.0±0.71	Moderate	GYC
15.	2.5	-	1.0	0.46±0.02	0.91±0.03	17.6±0.93	34.4±0.68	Few	YGC
16.	MS0 (Control)			0.44±0.02	0.78±0.03	14.2±0.86	27.2±0.86	Few	WGF

DAI = Days After Inoculation; Few (0 ≤ Few ≤ 49 no), Moderate (50 ≤ Moderate ≤ 69 no), Many (70 ≤ Many ≤ Above 70 no); Yellowish Green Compact (YGC), Yellowish Green Friable (YGF), Whitish Green Compact (WGC), Whitish Green Friable (WGF), Green Compact (GC), Greenish Friable (GF), Greenish Yellow Compact (GYC), Green Yellowish Friable (GYF); Values represent mean ± SE of each experiment consist of five replicates.

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

Fig. 2: Development of plantlets of *P. imbricata* on PM + 0.5mg/l BAP + 0.5mg/l NAA.

Fig. 3: Multiplication of *in vitro* derived PLBs of *P. imbricata* on MS + 2.0 mg/l 2, 4-D.

Fig. 4: Amplification of *in vitro* raised PLBs of *P. imbricata* on MS + 2.0 mg/l 2, 4-D + 0.8 mg/l BAP.

Discussion

The study confirms that nutritional composition plays a critical role in orchid seed germination. MS medium consistently outperformed PM, MVW and KC media. This superiority is likely attributed to the high concentration of nitrogen in the form of ammonium and nitrate in MS salts, which is essential for the rapid synthesis of proteins and nucleic acids during the early heterotrophic stage of orchid development [5]. Similar findings were reported for *Pholidota articulata*, where MS medium favored earlier protocorm differentiation compared to KC [9]. Briefly comparing the media, KC medium, which is often used for *Cymbidium*, proved too nutrient poor for the rapid development of *P. imbricata*, resulting in a 50% lower germination rate than MS.

The inclusion of PGRs (BAP and NAA) was crucial for breaking dormancy and accelerating differentiation. The balanced ratio of cytokinin (BAP) and auxin (NAA) facilitates cell division and elongation simultaneously. This aligns with findings in *Phaius tancarvilleae*, where BAP and NAA synergism significantly reduced the time required for protocorm formation [10].

For PLBs multiplication, the combination of 2,4-D and BAP proved highly effective. 2,4-D is a potent auxin known to induce stress mediated dedifferentiation (callus formation) or direct somatic embryogenesis in orchids [11]. However, 2,4-D alone can sometimes retard organogenesis. The addition of BAP counters this by promoting shoot bud differentiation from the 2,4-D induced meristematic clumps. This "Auxin-Cytokinin Synergism" is a well-documented phenomenon in orchid tissue culture; for instance, comparable results have been observed in *Vanda spathulata* and *Dendrobium* hybrids where dual hormone strategies maximized shoot multiplication [12-13]. In contrast, combinations involving kinetin (Kn) were less effective, suggesting that *P. imbricata* tissues have a higher specific sensitivity to benzyl adenine derivatives for shoot proliferation.

The 75% survival rate during hardening is consistent with epiphytic orchids, which are prone to desiccation shock when moved from the high humidity *in vitro* environment to *ex vitro* conditions. The use of porous substrates like coconut coir and coal helped maintain aeration while retaining moisture, mimicking the natural epiphytic habitat [14].

Conclusion

This research successfully established a reproducible protocol for the mass propagation of *Pholidota imbricata*. The study identifies MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA as the optimal condition for seed germination. Furthermore, a high-frequency PLBs multiplication system was developed using 2.0 mg/l 2,4-D and 0.8 mg/l BAP. This protocol can be directly applied to the conservation of this threatened medicinal orchid and its commercial production for pharmaceutical exploitation.

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Conflicts of interest

The authors declare that they have no conflicts of interest related to this publication.

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