



## ***In vitro* micropropagation of *Acampe ochracea* (Lindl.) Hochr. of Bangladesh: An efficient regeneration from nodal and leaf explants**

Marzia Akter Sumi, Awishik Tripura, Somaya Emrog Nayma, Minhajur Rahman, Tapash Kumar Bhowmik\*

Department of Botany, Faculty of Biological Sciences, University of Chittagong, Chattogram, Bangladesh

### **Abstract**

**Background:** *Acampe ochracea* (Lindl.) Hochr. is an epiphytic orchid of significant ornamental and medicinal value distributed across Bangladesh and Southeast Asia? Due to habitat destruction, over-collection and slow natural propagation rates, its population is declining. Conventional propagation methods are insufficient to meet commercial and conservation demands.

**Methods:** This study established an efficient *in vitro* micropropagation protocol using nodal and leaf segments excised from *in vitro* raised seedlings. Explants were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-Benzylaminopurine (BAP), Kinetin (Kn),  $\alpha$ -Naphthaleneacetic acid (NAA), Indole-3-acetic acid (IAA) and Picloram (Pic) to induce direct organogenesis and protocorm-like bodies (PLBs). Subsequent shoot elongation was evaluated in both solid and liquid MS media, followed by rooting in auxin enriched media and acclimatization.

**Results:** The highest frequency of multiple shoot buds (MSBs) from nodal segments ( $93.33 \pm 1.72\%$ ) with a maximum number of shoots ( $6.2 \pm 0.58$  per explant) was achieved on MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l NAA. Leaf segments produced the highest percentage of green PLBs ( $77.33 \pm 1.96\%$ ) on the same medium. For shoot elongation, solid MS medium fortified with 1.5 mg/l BAP + 0.9 mg/l NAA proved superior, yielding shoots of  $2.81 \pm 0.03$  cm. Rooting was optimal on MS medium supplemented with 0.5 mg/l IAA + 0.5 mg/l NAA, producing  $5.17 \pm 0.40$  roots per shoot. The survival rate of transplanted plantlets was 70% during acclimatization.

**Conclusion:** This optimized protocol provides a reliable method for the mass propagation of *A. ochracea*, facilitating both commercial exploitation and *ex situ* conservation efforts.

**Keywords:** *Acampe ochracea*, micropropagation, nodal explants, PLBs, acclimatization

### **Introduction**

Orchids, belonging to the family Orchidaceae, represent one of the most diverse and evolutionarily advanced groups of flowering plants, comprising approximately 26,567 species [1]. Beyond their horticultural significance as long-lasting cut flowers and potted plants, orchids possess substantial medicinal properties. The global floriculture industry has seen a compounded annual growth rate of over 10%, with orchids accounting for approximately 10% of the international fresh cut flower trade [2]. In Bangladesh, the family is represented by 188 species, many of which are epiphytic and reside in the forests of Chittagong Hill Tracts, Sylhet and the Sundarbans [3].

*Acampe ochracea* (Lindl.) Hochr. is a pendulous epiphytic monopodial orchid native to Bangladesh and the broader Indo-Malayan region. It is valued not only for its elegant yellow flowers with brown markings but also for its therapeutic potential. Recent studies have highlighted its hepatoprotective properties and antioxidant activity, attributed to its rich secondary metabolite profile [4, 5]. Ethnobotanical surveys indicate that orchid species in this region are frequently used in traditional medicine to treat various ailments, including rheumatism and fractures [6]. However, the natural population of *A. ochracea* is under severe threat due to anthropogenic activities, including deforestation, habitat fragmentation and indiscriminate collection for the horticultural trade [7]. Furthermore, natural propagation is hampered by the lack of endosperm in seeds, necessitating specific mycorrhizal associations for germination, resulting in slow growth rates in the wild [8].

To mitigate the risk of extinction and meet commercial demands, biotechnological interventions are essential. Plant tissue culture, specifically micropropagation, offers a viable solution for the rapid mass production of uniform, disease-free planting material [9]. Recent advances in orchid biotechnology have focused on optimizing protocols for recalcitrant species using various explants such as shoot tips, nodes and leaves [10]. While protocols exist for various orchid genera such as *Dendrobium* and *Phalaenopsis*, efficient regeneration systems for *Acampe* species using vegetative explants remain limited [11, 12]. Direct organogenesis from nodal and leaf explants is often preferred over callus-mediated pathways to maintain genetic fidelity and reduce somaclonal variation [13]. Recent reviews in Plant Cell, Tissue and Organ Culture emphasize the critical role of cytokinin-auxin synergism in regulating morphogenesis in monopodial orchids and the increasing use of advanced biotechnological tools for conservation [14, 15].

The present study aims to establish a reliable and efficient *in vitro* micropropagation protocol for *A. ochracea* using nodal and leaf explants. We investigated the effects of various Plant Growth Regulators (PGRs) on multiple shoot buds (MSBs) induction, Protocorm-Like Bodies (PLBs) formation, shoot elongation and rooting to facilitate mass propagation and conservation of this valuable species.

### **Materials and Methods**

#### **Plant material and explant preparation**

Mature capsules of *Acampe ochracea* were collected from Bandarban, CHT, Bangladesh. Aseptic seedlings were

raised *in vitro* to serve as the source of explants. Nodal segments (1.0-1.5 cm) and leaf segments (0.5-1.0 cm) were excised from these healthy, *in vitro* grown seedlings and used as explants for the micropropagation experiments.

### Media Preparation and Culture Conditions

Murashige and Skoog (MS) medium [16] supplemented with 3% (w/v) sucrose was used as the basal medium. The medium was solidified with 0.8% (w/v) agar (Fluka). The pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl prior to autoclaving at 121°C and 1.05 kg/cm<sup>2</sup> pressure for 20 minutes. Stock solutions of macronutrients, micronutrients, vitamins and PGRs were prepared separately. The cultures were incubated in a culture room maintained at 25 ± 2°C with a photoperiod of 14 hours light provided by cool-white fluorescent tubes (approx. 2000-3000 lux) and 10 hours dark.

### Micropropagation via Nodal and Leaf Explants

To standardize the optimal PGRs combination for shoot multiplication, nodal and leaf segments were cultured on MS medium supplemented with various concentrations of Cytokinins (BAP, Kinetin) and Auxins (NAA, IAA, Picloram). Data on the percentage of explants responding, time required for bud initiation and number of Multiple Shoot Buds (MSBs) or PLBs per explant were recorded after 4-6 weeks.

### Shoot Elongation

Micropropagated shoot buds often exhibit slow growth; therefore, they were transferred to specific elongation media. Both solid (0.8% agar) and liquid MS media supplemented with different concentrations of BAP, Kinetin and NAA were evaluated. The shoot length was measured initially and after 30 days of culture to determine the growth increase.

### In vitro Rooting

Elongated shoots (2-3 cm in length) were excised and transferred to rooting media comprising MS basal salts supplemented with various auxins: IAA, IBA and NAA, either individually or in combination. Parameters such as the number of roots per shoot and root length were recorded.

### Acclimatization and Transplantation

Well-rooted plantlets were subjected to a hardening process. The culture vessels were opened and kept in the culture room for 24 hours, followed by exposure to ambient room conditions for 3-5 days. The plantlets were then removed, washed to remove agar and planted in small plastic pots containing a mixture of brick chips, charcoal and coconut coir (1:1:1). They were maintained at room temperature for 3-5 days before transfer to the natural environment.

### Statistical Analysis

The experiments were set up in a completely randomized design with five replicates per treatment. Data were expressed as Mean ± Standard Error (SE).

### Results

#### Shoot regeneration from nodal and leaf explants

The morphogenic response of nodal and leaf explants varied significantly with the type and concentration of PGRs (Table 1). Nodal segments underwent direct organogenesis, producing Multiple Shoot Buds (MSBs). The highest regeneration frequency (93.33 ± 1.72%) and the maximum number of shoots (6.2 ± 0.58 per segment) were achieved on MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l NAA. This was achieved in the shortest time (5.20 ± 0.14 weeks; Figure 1). Leaf segments differentiated into Protocorm-Like Bodies (PLBs). The optimal response (77.33 ± 1.96% induction; Figure 2) was also observed on MS medium with 2.0 mg/l BAP + 1.0 mg/l NAA, producing green PLBs within 5.60 ± 0.12 weeks. Combinations of BAP and NAA generally outperformed kinetin-based treatments or single PGR applications.

#### Elongation of Shoots

The elongation of micropropagated shoots was evaluated in both solid and liquid MS media (Table 2). While both systems supported growth, solid media generally yielded sturdier shoots. The maximum shoot length (2.81 ± 0.03 cm) was recorded on solid MS medium supplemented with 1.5 mg/l BAP + 0.9 mg/l NAA (Figure 3). The corresponding liquid medium resulted in a length of 2.37 ± 0.03 cm (Figure 4). Synergistic use of BAP and NAA proved more effective for elongation than BAP alone.

**Table 1:** Development of MBSs/PLBs from *in vitro* raised nodal and leaf explants of *A. ochracea* on semi-solid 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.	-	1.0	-	-	-	18.67±1.44	7.44±0.11	1.6±0.40	0.00±0.00	0.00±0.00	-
4.	-	2.0	-	-	-	25.33±2.24	7.20±0.14	2.0±0.32	0.00±0.00	0.00±0.00	-
5.	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
6.	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
7.	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
8.	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
9.	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
10.	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
11.	-	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

12.	-	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
13.	-	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
14.	-	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
15.	-	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
16.	-	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
17.	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.

**Rooting and hardening**

Robust rooting is critical for transplantation success. Among the media tested, MS medium supplemented with 0.5 mg/l IAA + 0.5 mg/l NAA induced the highest number of roots (5.17 ± 0.40) and the greatest root length (4.31 ± 0.04 cm; Table 3 and Figure 5). Approximately 70% of the plantlets survived the acclimatization process and established successfully in the potting mixture (brick, coal, coconut coir) under natural environmental conditions.

**Table 2:** Elongation of MSBs derived shoot bud developed from nodal explant of *A. ochracea* on agar solidified and liquid MS medium with different kinds of PGRs.

Sl. No.	PGR Conc. (mg/l)				Solid media			Liquid media		
	BAP	Kn	NAA	IAA	Initial length (cm) of shoot bud (Mean± SE)	Length (cm) of shoot bud after 30d of culture (Mean± SE)	Increased in length (cm) of shoot bud within 30d of culture (Mean± SE)	Initial length (cm) of shoot bud (Mean± SE)	Length (cm) of shoot bud after 30d of culture (Mean± SE)	Increased in length (cm) of shoot bud within 30d of culture (Mean± SE)
1.	1.0	-	-	-	1.39±0.02	3.58±0.04	2.19±0.03	1.33±0.02	3.21±0.05	1.87±0.03
2.	1.5	-	-	-	1.36±0.02	3.79±0.05	2.43±0.03	1.36±0.02	3.44±0.03	2.08±0.04
3.	2.0	-	-	-	1.35±0.03	3.64±0.07	2.28±0.04	1.27±0.02	3.18±0.05	1.92±0.03
4.	-	1.0	-	-	1.29±0.02	3.33±0.03	2.04±0.03	1.31±0.02	3.05±0.02	1.74±0.03
5.	-	1.5	-	-	1.37±0.03	3.46±0.04	2.09±0.02	1.26±0.02	3.08±0.03	1.82±0.03
6.	-	2.0	-	-	1.34±0.03	3.40±0.03	2.06±0.03	1.30±0.02	3.07±0.03	1.77±0.03
7.	1.0	-	0.6	-	1.38±0.02	4.04±0.02	2.65±0.03	1.37±0.02	3.57±0.04	2.20±0.03
8.	1.5	-	0.9	-	1.35±0.03	4.16±0.05	2.81±0.03	1.32±0.02	3.69±0.02	2.37±0.03
9.	2.0	-	1.2	-	1.33±0.03	4.05±0.01	2.72±0.03	1.28±0.02	3.56±0.04	2.28±0.02
10.	1.0	-	-	0.6	1.33±0.02	3.73±0.04	2.39±0.04	1.30±0.02	3.42±0.05	2.11±0.03
11.	1.5	-	-	0.9	1.29±0.02	3.97±0.05	2.68±0.04	1.27±0.02	3.51±0.02	2.25±0.03
12.	2.0	-	-	1.2	1.31±0.03	3.88±0.05	2.57±0.03	1.32±0.03	3.50±0.06	2.18±0.04
13.	-	1.0	0.6	-	1.37±0.03	3.68±0.01	2.31±0.03	1.37±0.02	3.41±0.03	2.03±0.03
14.	-	1.5	0.9	-	1.34±0.03	3.95±0.06	2.61±0.03	1.30±0.02	3.53±0.03	2.23±0.03
15.	-	2.0	1.2	-	1.38±0.03	3.88±0.02	2.50±0.04	1.35±0.02	3.48±0.04	2.13±0.03
16.	-	1.0	-	0.6	1.32±0.02	3.58±0.05	2.26±0.03	1.26±0.01	3.24±0.03	1.98±0.03
17.	-	1.5	-	0.9	1.38±0.02	3.92±0.03	2.54±0.03	1.35±0.03	3.51±0.05	2.16±0.04
18.	-	2.0	-	1.2	1.31±0.01	3.78±0.04	2.47±0.04	1.36±0.02	3.41±0.04	2.05±0.04
19.	MS0 (Control)				1.35±0.02	3.24±0.04	1.88±0.04	1.29±0.03	2.85±0.03	1.56±0.03

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

**Table 3:** Mean increase in length (cm) and number of roots per MSBs derived seedlings of *A. ochracea* in auxin supplemented MS and MS0 rooting media

Sl. No.	PGR Concentration (mg/l)			Initial length (cm) per MSBs derived seedling (Mean ± SE)	Initial number of roots per MSBs derived seedling (Mean ± SE)	Increased length (cm) per MSBs derived seedling (Mean ± SE)	Increased number of roots per MSBs derived seedling (Mean ± SE)
	IAA	IBA	NAA				
1.	0.5	-	-	1.47±0.03	1.00±0.26	3.27±0.06	4.33±0.49
2.	1.0	-	-	1.49±0.03	0.67±0.21	3.83±0.05	3.33±0.33
3.	-	0.5	-	1.41±0.02	1.17±0.31	3.39±0.06	4.00±0.37
4.	-	1.0	-	1.45±0.02	0.67±0.21	3.06±0.08	3.67±0.33
5.	-	-	0.5	1.52±0.02	1.17±0.31	3.63±0.07	4.33±0.33
6.	-	-	1.0	1.48±0.04	0.67±0.33	3.14±0.07	3.83±0.40
7.	0.5	0.5	-	1.46±0.03	1.17±0.31	3.78±0.06	4.50±0.43
8.	1.0	1.0	-	1.43±0.03	0.83±0.31	3.50±0.07	4.17±0.48
9.	-	0.5	0.5	1.48±0.04	1.00±0.37	4.13±0.09	5.00±0.37
10.	-	1.0	1.0	1.43±0.03	1.17±0.31	3.92±0.08	4.50±0.43
11.	0.5	-	0.5	1.44±0.03	0.67±0.21	4.31±0.04	5.17±0.40
12.	1.0	-	1.0	1.42±0.02	0.83±0.31	4.05±0.07	4.83±0.40
13.	MS0 (Control)			1.47±0.03	1.17±0.17	2.03±0.04	2.33±0.21

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



**Fig 1:** Development of MSBs in *A. ochracea* sprouted from *in vitro* raised nodal segment on MS + 2.0 mg/l BAP + 1.0 mg/l NAA



**Fig 2:** Development of PLBs in *A. ochracea* sprouted from *in vitro* elevated leaf segment on MS + 2.0 mg/l BAP + 1.0 mg/l NAA



**Fig 3:** An individual seedling of *A. ochracea* undergoing elongation on MS + 1.5 mg/l BAP + 0.9 mg/l NAA



**Fig 4:** Seedling of *A. ochracea* undergoing elongation in liquid MS + 2.0 mg/l BAP + 1.2 mg/l NAA



**Fig 5:** Induction of strong and stout root system in MSBs derived seedlings of *A. ochracea* on MS + 0.5 mg/l IAA + 0.5 mg/l NAA

### Discussion

The conservation of endangered orchid species through micropropagation requires the optimization of explant type and PGRs balance. In this study, nodal segments of *Acampe ochracea* proved to be superior explants for multiple shoot induction compared to leaf segments. This aligns with findings in other monopodial orchids like *Vanda* and *Aerides*, where axillary buds in nodal segments allow for rapid direct organogenesis [17]. The efficiency of nodal segments has been further validated by recent studies on *Phalaenopsis*, where nodal explants yielded high regeneration rates without callus intermediates, ensuring genetic fidelity [18].

The synergistic effect of cytokinins (BAP) and auxins (NAA) was evident in this study. The combination of 2.0 mg/l BAP and 1.0 mg/l NAA yielded the highest shoot multiplication. BAP is widely recognized as the most effective cytokinin for orchid micropropagation, often enhancing cell division in meristematic zones [19]. Recent research has consistently highlighted that a balanced ratio of BAP and NAA is crucial for breaking apical dominance and inducing PLBs in recalcitrant orchid species [14, 20]. High concentrations of BAP alone can sometimes lead to stunted growth or hyperhydricity, but the addition of NAA appears to mitigate these effects and promote healthy shoot proliferation [21]. This synergistic mechanism is supported by findings in *Dendrobium transparens*, where similar combinations maximized PLBs induction and shoot differentiation [22].

Leaf explants exhibited a high capacity for PLBs formation (77.33%) on the same BAP and NAA fortified medium. The induction of PLBs from somatic tissues is a preferred pathway for mass propagation as it mimics zygotic embryogenesis. This finding is consistent with results reported for *Phalaenopsis*, where leaf segments cultured on thidiazuron or BAP or NAA combinations successfully regenerated PLBs *via* somatic embryogenesis [23, 24]. Furthermore, recent advancements in temporary immersion systems suggest that scaling up such protocols can significantly reduce production costs while maintaining plant quality [25].

For shoot elongation, solid media performed better than liquid media in this study. While liquid culture often facilitates nutrient uptake, it can sometimes cause hyperhydricity in orchids [26]. The use of solid MS with 1.5 mg/l BAP + 0.9 mg/l NAA provided a stable matrix for structural development. Recent economic analyses also

suggest that while bioreactors are efficient, solid media remains reliable for smaller-scale high-fidelity conservation projects [27].

Rooting was most effective with a combination of IAA and NAA. While IBA is often the standard for rooting, the combination of IAA and NAA (0.5 mg/l each) provided a synergistic effect, enhancing both root number and length. This contrasts with some studies where IBA alone was sufficient, suggesting species-specific auxin requirements for *Acampe* [28, 29]. The 70% survival rate during acclimatization is promising but suggests room for improvement in humidity control during the initial *ex vitro* transfer. Emerging strategies using endophytes or specific potting substrates, could further enhance these survival rates in future trials [30].

### Conclusion

The present study establishes a reproducible and efficient protocol for the micropropagation of *Acampe ochracea* using nodal and leaf explants. The combination of BAP and NAA was identified as the critical hormonal signal for both direct shoot organogenesis and PLB induction. This protocol offers a sustainable method for the mass production of this commercially and medicinally important orchid, thereby reducing pressure on natural populations and contributing to the biodiversity conservation of Bangladesh's orchid flora. Future research should integrate molecular markers to assess the genetic fidelity of regenerates and explore the use of elicitors to enhance secondary metabolite production for pharmaceutical applications.

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### Conflicts of Interest

The authors declare that they have no conflicts of interest in relation to the publication of this manuscript.

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