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In vitro Seed Germination and Root Induction of *Arundina graminifolia* (D. Don.) Hochr. A Native Orchid in Chittagong Hill Tracts of Bangladesh

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Abstract

Arundina graminifolia (D. Don.) Hochr., commonly known as the bamboo orchid, is a terrestrial orchid of significant medicinal and ornamental value native to the Chittagong Hill Tracts (CHT) of Bangladesh. To establish an efficient conservation strategy, *in vitro* asymbiotic seed germination and successful root induction protocols were developed. Mature green capsules were cultured on four basal media *viz*. Knudson C (KC), MS, Phytamax (PM) and Vacin and Went (VW), at both half and full strengths, with or without plant growth regulators (PGRs). Full-strength PM medium supplemented with 0.5 mg/l 6-Benzylaminopurine (BAP) and 0.5 mg/l α-Naphthalene acetic acid (NAA) produced the highest germination percentage (93.34 ± 1.96%) and the shortest initiation time (6.21 ± 0.22 weeks). Subsequent seedling development, including protocorm formation and differentiation of leaf and root primordia, was significantly faster on this medium compared to MS, VW or KC. For the induction of a robust root system, elongated seedlings were transferred to half-strength MS medium supplemented with various auxins. The combination of 1.0 mg/l Indole-3-butyric acid (IBA) and 1.0 mg/l NAA proved most effective, producing the maximum number of roots (4.17 ± 0.31) and root length (3.91 ± 0.04 cm) within 30 days. Following a stepwise acclimatization process, 81.67% of the seedlings survived and were successfully established in the *ex vitro* environment. Statistical analysis confirmed the superiority of PM for germination and of auxin-supplemented ½MS for rooting. This protocol provides an effective method for mass propagation of this orchid.

Introduction

The Orchidaceae family, comprising approximately 28,237 species, represents one of the most advanced and diverse groups of angiosperms (Willis 2017). Orchids are renowned not only for their exquisite floral architecture but also for their profound medicinal

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properties and economic value in the global floriculture market (De and Pathak 2020, Thammasiri 2020). While the majority of orchids are epiphytic (70%), a significant portion (25%), including *Arundina graminifolia* (D. Don.) Hochr., are terrestrial (Fischer et al. 2011).

A. graminifolia, well-known as the bamboo orchid due to its reed-like stems and grass-like leaves, is native to tropical Asia, including the Chittagong Hill Tracts (CHT) of Bangladesh. Beyond its ornamental appeal, it holds substantial ethnopharmacological importance. The roots are traditionally used to treat snakebites, rheumatism and intestinal colic, while root pastes are applied to alleviate pain and swelling (Huda et al. 2006, Hossain 2009). Recent studies have also highlighted its potential in modern pharmacology due to the presence of bioactive compounds such as stilbenoids and phenanthrenes (Singh 2022). Various alkaloids have been identified (Zhang et al. 2021) and extracts show antioxidant, antimicrobial and anticancer activities. However, *A. graminifolia* populations are declining due to habitat loss and overharvest; it is considered a threatened species (Verma et al. 2012)

Despite its value, the natural propagation of *A. graminifolia* is hindered by the unique reproductive biology of orchids. Orchid seeds are minute, lack endosperm, and require a symbiotic relationship with specific mycorrhizal fungi to germinate in the wild (Bernard 1909). Consequently, germination rates in nature are perilously low (Swarts and Dixon 2009). This physiological bottleneck, compounded by habitat destruction in the CHT region, necessitates urgent *ex situ* conservation measures.

In vitro culture techniques offer a viable solution for the mass propagation and conservation of threatened orchid species (Bhowmik et al. 2024). While protocols exist for various orchids, species-specific optimization of nutrient media and plant growth regulators (PGRs) is critical for success (Bhowmik and Rahman 2022). Previous studies have utilized MS and Phytamax (PM) media for diverse species, yet a standardized protocol for the CHT germplasm of *A. graminifolia* focusing on rapid seed germination and robust rooting remains underexplored.

The objective of this study was to develop a reliable, reproducible protocol for the *in vitro* asymbiotic seed germination, differentiation and root induction of *A. graminifolia*, facilitating its conservation and further sustainable utilization.

Materials and Methods

Mature green capsules of *A. graminifolia* were collected from natural habitats in Ruma Upazilla, Bandarban, Bangladesh. The capsules were washed under running tap water for 10 minutes to remove surface debris. Surface sterilization was performed in a laminar air flow cabinet. Capsules were treated with 70% (v/v) ethanol for 30 sec, followed by immersion in 3% (w/v) sodium hypochlorite (NaOCl) for 10 min. Finally, they were submerged in a 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10 min and rinsed three times with sterile double-distilled water.

Four basal media were evaluated for seed germination: Knudson C, KC (Knudson 1946); MS; Phytamax, PM (Arditti 1977) and Vacin and Went, VW (Vacin and Went 1949). Each medium was tested at half and full strength, both with and without the supplementation of 0.5 mg/l BAP and 0.5 mg/l NAA. All media were gelled with 0.8% (w/v) agar (HiMedia, India) and the pH was adjusted to 5.8 for MS and 5.4 for KC, PM and VW before autoclaving at 121°C for 20 min. Cultures were incubating at 25 ± 2°C under a 14 hrs photoperiod provided by white fluorescent tubes (3500-5000 lux).

Sterilized capsules were dissected longitudinally and seeds were inoculated onto the prepared media. Data were recorded on the initiation of germination, protocorm development and the differentiation of leaf and root primordia. The percentage of germination was calculated based on the number of culture vessels showing a response.

Elongated seedlings (2-3 cm height) derived from the germination phase were transferred to half-strength MS medium supplemented with various concentrations of auxins: Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and NAA, either individually or in combination. The number and length of roots were recorded after 30 days of culture.

Ninety-day-old seedlings with well-developed roots were subjected to a stepwise hardening process. They were transferred to pots containing a mixture of sterilized soil, sand, activated charcoal and pit moss (1 : 1 : 1 : 1) and maintained in a greenhouse at 25-30°C with 60-70% relative humidity.

The experiment followed a Completely Randomized Design (CRD). Data were analyzed using ANOVA and mean separation was performed using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ using SPSS software.

Results and Discussion

The germination response of *A. graminifolia* seeds varied significantly depending on the basal media and strength used (Table 1, Fig. 1a-1h). The initiation of germination was visibly marked by the swelling of the embryo and the subsequent rupture of the testa, leading to the formation of protocorm-like bodies (PLBs).

Seed germination of *A. graminifolia* was highest (93.34%) in full-strength PM medium combined with PGRs, followed by PGRs enhanced full-strength MS (86.67%, Fig. 1a), VW (66.67%, Fig. 1b) and KC (60.00%) media, respectively. Responses for seed germination in half-strength PGRs-free KC medium were the lowest, at 26.67%. The minimum time required for the initiation of seed germination was found to be on PGRs supplemented full-strength PM (6.21 ± 0.22 w) medium, followed by MS (7.12 ± 0.11 w), VW (9.57 ± 0.18 w) and KC (10.23 ± 0.25 w) media, respectively. In contrast, the duration required for seed germination initiation (15.13 ± 0.22 w), protocorm development (21.20 ± 0.30 w, Fig. 1c), first leaf primordia differentiation (27.17 ± 0.30 w), first root primordia differentiation (34.23 ± 0.27 w) and seedling development (42.20 ± 0.35 w) in PGRs free half-strength KC medium was the longest. Post seed germination, the shortest duration required for

protocorm development (10.65 ± 0.27 w, Fig. 1d), first leaf primordium differentiation (14.41 ± 0.21 w), first root primordium differentiation (20.97 ± 0.29 w) and seedling development (24.82 ± 0.34 w, Fig. 1e) occurred on PGRs enriched full-strength PM medium followed by MS medium (11.62 ± 0.25; 15.28 ± 0.27; 21.90 ± 0.22; 26.65 ± 0.34 w, Fig. 1f) respectively.

Table 1. Effects of different strength of KC, MS, PM and VW media with or without PGRs on *in vitro* seed germination, differentiation and seedlings development of *A. graminifolia*.

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	Half without PGRs	15.13 ± 0.22^{i}	21.20 ± 0.30^{i}	27.17 ± 0.30^{h}	34.23 ± 0.27^{i}	42.20 ± 0.35^{i}	26.67	+
	Full without PGRs	13.77 ± 0.15^{g}	18.33 ± 0.22^{g}	24.56 ± 0.32^{f}	30.73 ± 0.28^{g}	38.63 ± 0.31^{h}	40.00	+
	Full of PGRs	10.23 ± 0.25^{d}	14.50 ± 0.20^{c}	19.47 ± 0.33^{c}	25.33 ± 0.27^{c}	32.03 ± 0.29^{d}	60.00	++
MS	Half without PGRs	12.30 ± 0.24^{f}	16.24 ± 0.21^{e}	22.60 ± 0.30^{e}	29.17 ± 0.24^{f}	37.20 ± 0.28^{g}	46.67	+
	Full without PGRs	8.07 ± 0.13^{b}	12.57 ± 0.20^{b}	17.23 ± 0.23^{b}	23.87 ± 0.25^{b}	29.60 ± 0.37^{b}	73.34	++
	Full of PGRs	7.12 ± 0.11^{b}	11.62 ± 0.25^{b}	15.28 ± 0.27^{b}	21.90 ± 0.22^{b}	26.65 ± 0.34^{b}	86.67	+++
PM	Half without PGRs	11.33 ± 0.21^{e}	15.17 ± 0.22^{d}	19.60 ± 0.35^{c}	26.60 ± 0.33^{d}	32.53 ± 0.25^{d}	53.34	++
	Full without PGRs	7.82 ± 0.24^{b}	11.94 ± 0.28^{b}	15.43 ± 0.37^{b}	22.07 ± 0.32^{b}	27.03 ± 0.28^{b}	80.00	+++
	Full of PGRs	6.21 ± 0.22^{b}	10.65 ± 0.27^{b}	14.41 ± 0.21^{b}	20.97 ± 0.29^{b}	24.82 ± 0.34^{b}	93.34	+++
VW	Half without PGRs	15.03 ± 0.20^{i}	20.23 ± 0.18^{h}	26.17 ± 0.33^{g}	32.23 ± 0.30^{h}	39.20 ± 0.22^{h}	33.34	+
	Full without PGRs	12.10 ± 0.28^{f}	16.07 ± 0.25^{e}	21.77 ± 0.25^{d}	28.20 ± 0.24^{e}	34.27 ± 0.25^{e}	46.67	+
	Full of PGRs	9.57 ± 0.18^{c}	14.33 ± 0.25^{c}	19.27 ± 0.22^{c}	25.07 ± 0.28^{c}	30.57 ± 0.30^{c}	66.67	++

PGRs (0.5 mg/l BAP + 0.5 mg/l NAA); '-' Indicate no response, + = Minimum germination (0% ≤ + ≤ 49%), ++ = Medium germination (50% ≤ ++ ≤ 74%), +++ = Maximum germination (75% ≤ +++ ≤ 100%). Values represent mean ± SE of each experiment consist of 15 replicates. Mean values followed by different superscript letters within a column are significantly different at p = 0.05 according to DMRT.

During the beginning of seed germination, there were significant differences ($p < 0.05$) among media of varying strengths, both with and without PGRs, except for the PGRs free full-strength MS media and the PGRs fortified full-strength VW media. PGRs are free of half-strength PM and full-strength MS; The development of protocorms on free full-strength PM and full-strength PGRs supplemented MS media showed no significant differences ($p < 0.05$). To differentiate the first leaf primordia, use half-strength PM without PGRs and full-strength VW; The PGRs free full-strength PM, PGRs fortified full-

strength MS, and VW media exhibited insignificant variation ($p < 0.05$). PGRs free half-strength PM and full-strength VW; The differentiation of first root primordia showed no significant differences ($p < 0.05$) between PGRs free full-strength PM and PGRs supplemented full-strength MS media. Different strengths, whether or not PGRs were included and supplemented with various media, demonstrated significant differences ($p < 0.05$) in seedling development.

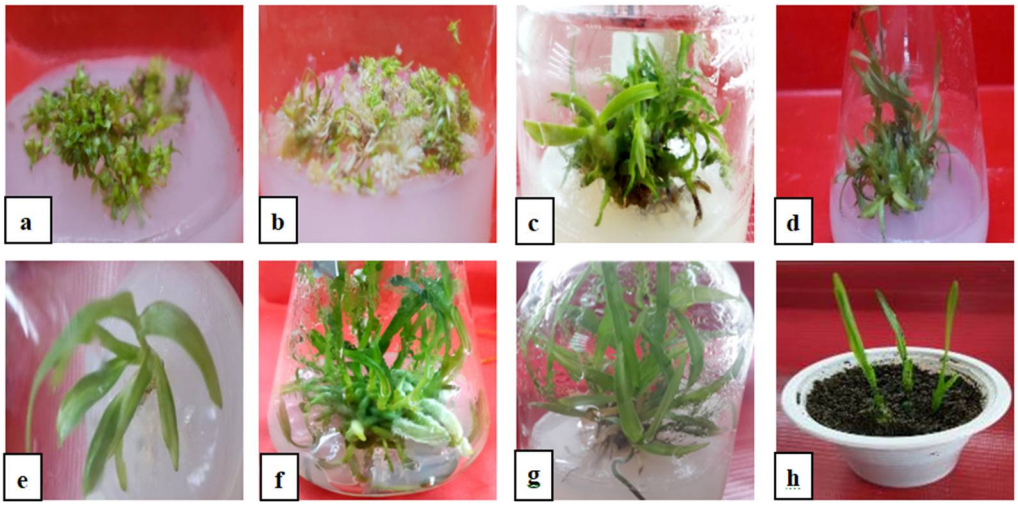
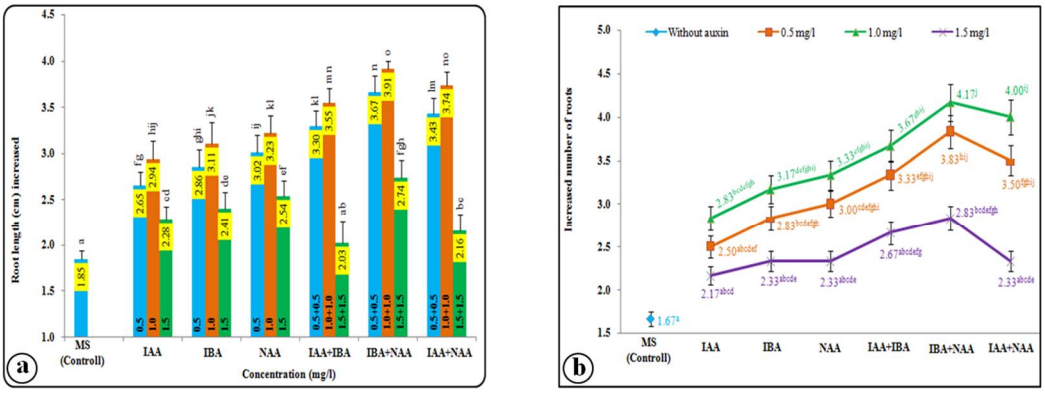


Fig. 1(a-h). Different stages of *A. graminifolia*: seedling development, *in vitro* rooting and acclimatization: (a) seed germination on full-strength MS medium, (b) seed germination on full-strength VW medium, (c) germinated PLBs turned into small shoots on full-strength KC medium, (d) germinated PLBs turned into small shoots on full-strength PM medium, (e) development of plantlet on PM + 0.5 mg/l BAP + 0.5 mg/l NAA, (f) seedlings developed on MS + 0.5 mg/l BAP + 0.5 mg/l NAA, (g) development of root system on ½MS + 1.0 mg/l IBA + 1.0 mg/l NAA, and (h) *in vitro* developed *A. graminifolia* seedling growing in pot outside of the culture room.

Seedlings germinated on the primary media and developed spontaneous roots, but these were typically thin and fragile. Upon transfer to half-strength MS medium supplemented with various auxins, a significant improvement in root morphology and number was observed (Fig. 2a and 2b). Half-strength MS medium with the combination of 1.0 mg/l IBA and 1.0 mg/l NAA proved to be the most effective treatment for root induction (Fig. 1g). This combination produced the maximum average root length (3.91 ± 0.04 cm) and the highest mean number of roots (4.17 ± 0.31) per plantlet after 30 days of culture. These values were significantly higher ($p \leq 0.05$) than those obtained from the control (MS0) or treatments using single auxins at lower concentrations.

Following the *in vitro* rooting phase, seedlings were subjected to a hardening process. After two months of maintenance in the greenhouse, a survival rate of 81.67% was recorded. The surviving plantlets exhibited healthy vegetative growth and established well (Fig. 1h) in the *ex vitro* conditions.



Values represent mean ± SE of each experiment consist of six replicates. Mean values of each point of a graph followed by different superscript letters are significantly different at $p \leq 0.05$ according to Duncan's Multiple Range Test.

Fig. 2(a-b). Seed-originated plantlets of *A. graminifolia* in auxin supplemented half-strength MS and MS0 rooting media after 30 d of culture: (a) increased root length (cm) and (b) increased root number.

The success of *in vitro* orchid conservation relies heavily on identifying the optimal nutritional requirements for specific species. In the present study, *A. graminifolia* exhibited a clear preference for PM medium over MS, VW or KC media. The superiority of PM medium for orchid seed germination has been well-documented in other species, including *Dendrobium transparens* (Bhowmik and Rahman 2020) and *Acampe rigida* (Bhowmik and Rahman 2021). This enhanced performance is likely attributed to the specific formulation of PM, which is enriched with vitamins and organic additives such as peptone. Peptone provides a readily available source of amino acids and nitrogen, which has been reported to significantly enhance seed germination and early protocorm development (Bhowmik and Rahman 2017).

The addition of PGRs played a crucial role in accelerating germination and development. The combination of BAP (a cytokinin) and NAA (an auxin) significantly reduced the time required for germination initiation compared to basal media alone. This finding aligns with studies on *Rhynchostylis retusa* (Thakur and Pathak 2021) and *Esmeralda clarkei* (Paudel et al. 2012), where the synergistic effect of cytokinins stimulating cell division and auxins promoting cell elongation was found to be essential for breaking dormancy and fostering early growth in orchid embryos.

For root induction, the results highlighted the necessity of auxins for developing a functional root system capable of supporting the plant *ex vitro*. While single auxins like NAA or IBA improved rooting compared to the control, the combination of IBA and NAA with half-strength MS medium yielded the superior results. This synergistic effect is consistent with findings in *Vanda tessellata* (Rahman et al. 2009, Aditya et al. 2020) and *Cymbidium aloifolium* (Paul et al. 2019). It is generally understood that IBA is highly effective at initiating root primordia, while NAA contributes to the subsequent thickening and elongation of the roots (Bhowmik and Rahman 2024). The robust root

system developed under this combined treatment likely contributed to the high survival rate (81.67%) observed during acclimatization, validating this protocol as an effective tool for the conservation of this valuable terrestrial orchid.

This study establishes a highly efficient protocol for the asymbiotic seed germination and root induction of the native *A. graminifolia* from the Chittagong Hill Tracts, Bangladesh. Full-strength PM medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA is recommended for rapid germination and protocorm development. For rooting, half-strength MS medium fortified with 1.0 mg/l IBA and 1.0 mg/l NAA ensures the production of robust seedlings suitable for transplantation. This protocol holds significant potential for the large-scale propagation and conservation of this valuable medicinal orchid.

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