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In vitro Seed Germination and Seedling Development of the Orchid *Acampe rigida* (Buch.-Ham. ex Sm.) P.F. Hunt

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Abstract

This study established an efficient *in vitro* seed germination and seedling development protocol for large-scale seedling production of *Acampe rigida*. Seed germination was highest on half strength MS medium (91.67%), followed by MS with 10% coconut water (83.33%), while lower rates were recorded on full strength MS medium (75%), Vacin and Went (VW) medium (58.33%) and Knudson's C (KC) medium (41.67%). Half-strength MS medium also showed the shortest time requirements for seed germination (17-22 days), protocorm-like body (PLB) development (30-35 days) and first leaf primordia differentiation (47-52 days). Compared to MS and ½MS, VW medium was suitable for the further development of protocorms. Protocorms exhibited 100% survival and seedling differentiation, with a maximum seedling length of 2.59 ± 0.10 cm in VW medium containing 10% coconut water and 10 g/l banana homogenate. Hormone-free VW medium with 10 g/l banana homogenate and 0.1% activated charcoal provides optimal growth and development of *in vitro* germinated seedlings. Healthy seedlings exhibited 80% survivability on a potting mixture of coconut husk, tree bark, charcoal and moss (2 : 1 : 1 : 1).

Introduction

The Orchidaceae is one of the most ecologically and morphologically diverse families of flowering plants with 880 genera and about 26,567 species (Cai et al. 2015). Except for Antarctica, every continent has members of this family, with Southeast Asia, South America and Central America having the greatest diversity (Dressler 1990). Bangladesh is also abundant in orchids with 188 species in 72 genera, of which 117 species (62.23%) are epiphytic and 71 species (37.77%) are terrestrial in nature. They are distributed throughout the country especially in Chittagong Hill Tracts, Cox's Bazar, Rangamati, greater Sylhet, Gazipur and Sundarban mangrove forest (Rahman et al. 2017).

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A. rigida is a well-known monopodial, epiphyte and rarely lithophyte species of orchid with outstanding floricultural potential. It is a robust species with a 60-90 cm stem and has lovely, fragrant pale yellow flowers with transverse crimson bands (Fig. 1a) which bloom during rainy season (Saikia and Borua 2016). This orchid is native to tropical Southern Asia and is mostly found in Cox's Bazar (Ramu) and Sundarban, Bangladesh (Rahman et al. 2017). They are generally cultivated for beautiful flowers and very less for their medicinal use. Capsules of this orchid consist of innumerable wind-dispersed microscopic seeds without endosperm and have an extremely low germination rate in nature of about 0.2-0.3% (Singh 1992, Rahman et al. 2013). These difficulties in natural germination and slow vegetative propagation, as well as severe deforestation and overexploitation for different purposes, may drive this orchid species to extinction, just like many other wild orchid species in our country (Rahman et al. 2013). According to the Plant Red List of Bangladesh: Volume 2 (2024), *A. rigida* is classified as Endangered (EN) within the country (IUCN Bangladesh 2024).

Thus, *in vitro* seed germination provides an effective solution for rapid seedling production, aiding the conservation of this beautiful species. This study developed a successful protocol for large-scale seedling production of *A. rigida* through *in vitro* seed germination and seedling development to conserve and commercialize this species.

Materials and Methods

In this experiment, fresh and indehiscent yellowish-green capsules (Fig. 1b) of *A. rigida* were used to initiate and develop the *in vitro* culture. The capsules were carefully rinsed under running tap water to remove dust particles and then with sterile distilled water using Tween-20. Further the capsules were surface sterilized with 0.2% $HgCl_2$ for 4-5 min, followed by a brief dipping in 100% ethanol and 2-3 times burning. Disinfected capsules were split longitudinally and seeded on various basal media: MS, half-strength MS (half-strength of MS macro- and micronutrients), KC (Knudson's C 1946), VW (Vacin and Went 1949) and MS with 10% coconut water (CW) to assess their effects on seed germination and protocorm development. The cultures were then be inoculated at 25 ± 1°C with a photoperiod of 16 h at 2000-3000 lux light intensity. The percentage of culture vessels that germinated in different strengths of basal media was calculated using the formula described by Bhowmik and Rahman (2022). Days required for onset of seed germination, protocorm-like bodies (PLBs) formation and 1st leaf primordia differentiation during asymbiotic seed culture of *A. rigida* were also calculated.

Protocorms with 1st leaf primordia and rhizoids were cultured on MS, ½MS and VW media containing 0.5 mg/l BAP, 10% CW and 10 g/l BH to investigate their effects on protocorm survival and seedling differentiation. Individual seedlings with 1-2 leaves were transferred to ½MS and VW media containing BAP, coconut water (CW), banana homogenate (BH) and activated charcoal (AC) to investigate their effect on seedling growth and development. Each experiment was repeated twice with five culture bottles

per replicate and 15 protocorms/ seedlings were inoculated into each vessel. Cultures were regularly sub-cultured at suitable intervals. Healthy seedlings with 4-5 leaves were removed from the culture vessels and acclimatized in a potting mixture containing coconut husk, tree bark, charcoal and moss (2 : 1 : 1 : 1). The potted plants were covered with transparent polythene bags and misted every 8 hours to maintain high humidity and prevent sudden desiccation. The plantlets were progressively exposed to the outer normal environment by gradually puncturing the polythene bags, which were then removed after a month, allowing the plantlets to establish properly.

Results and Discussion

Among the 5-types of germination media (Table 1), ½MS medium gave the topmost response (91.67%) of seeds germination in *A. rigida* (Fig. 1c, e-h), followed by MS + 10% CW (83.33%) (Fig. 1d), MS (75%) and VW (58.33%) media. Poor germination rate was observed on KC medium (41.67%). The lowest required time for onset of seed germination was also recorded on ½MS medium (17-22 d) followed by MS + 10% CW (22-27 d), VW (25-30 d) and MS (26-31 d) media. KC medium needed the utmost time (30-35 d) for onset germination (Table 1). ½MS medium exhibited the minimum time required for PLBs formation (30-35 d) and differentiation of 1st leaf primordia (47-52 d), while KC medium needed the maximum time for PLBs formation (47-52 d) and differentiation of 1st leaf primordia (36-68 d) (Table 1). Similar studies were conducted by Ragu et al. (2022) on *Paphiopedilum lowii*, Kim et al. (2021) on *Pelatantheria scolopendrifolia*, An et al. (2021) on *Sedirea japonica*, Sorgato et al. (2020) on *Dendrobium* and Pebam et al. (2016) on *Vanda stangeana*, and they discovered that ½MS medium was the most effective for seed germination and PLBs formation.

Table 1. Effects of different basal media on onset of seed germination, PLBs formation and 1st leaf primordia differentiation during asymbiotic seed culture of *A. rigida.*

Basal Medium	Required time in days			% of culture vessels where seeds germinated	Remarks
	Onset germination	PLBs formation	1st leaf primordia differentiation		
MS	26-31	42-47	57-62	75.00	++
½ MS	17-22	30-35	47-52	91.67	++++
MS + 10% CW	22-27	35-40	50-55	83.33	+++
KC	30-35	47-52	63-68	41.67	+
VW	25-30	45-50	60-65	58.33	+

For each treatment, 12 culture bottles were used; germination frequency: + = low, ++ = medium, +++ = good, ++++ = maximum. KC = Knudson's C; VW = Vacin and Went; MS = Murashige and Skoog; PLBs: Protocorm like bodies.

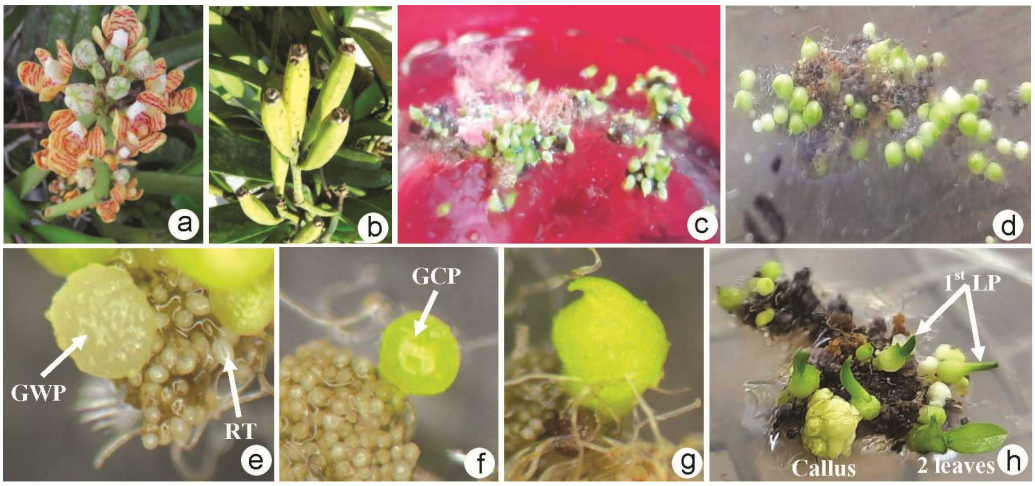

Fig. 1 (a-h). *In vitro* seed germination and protocorm development of *A. rigida*: (a) *A. rigida* flowering plant, (b) Indehiscent yellowish-green capsules (c-d) PLBs formation in, (c) ½MS and, (d) MS + 10% CW (e-g) Different stages of seed germination, (e) ruptured testa (RT) and globular white protocorms (GWP), (f) globular chlorophyllous protocorm (GCP), (g) protocorm with pointed shoot apex and rhizoids, (h) Protocorm with 1st leaf primordial (1st LP), two leaves and callus.

Protocorms exhibited 100% survivability in the zero ½MS medium, as well as in the ½MS and VW media containing 10% CW and 10 g/l BH (Table 2). In contrast, MS medium, with or without growth regulator and additives, did not improve protocorm survivability. However, compared to the other medium combinations, the VW medium with 10% CW and 10 g/l BH (100%) (Fig. 2 a-c) and ½MS medium with 10% CW and 10 g/l BH (93.33%) showed a significantly higher percentage of seedling differentiation.

Table 2. Effects of different basal media and additives on protocorms survival and seedling differentiation, following 8-weeks of the first sub-culture.

Media composition				Survival (%)	Seedlings differentiation (%)	Seedlings length (cm) (Mean ± SE)
Basal Medium	CW (%)	BH (g/l)	BAP (mg/l)			
½MS	-	-	-	100	80.00	1.85 ± 0.08
½MS	10	10	-	100	93.33	2.18 ± 0.12
MS	-	-	-	60.00	26.67	1.37 ± 0.09
MS	10	-	-	73.33	40.00	1.52 ± 0.13
MS	10	-	0.5	73.33	53.33	1.60 ± 0.09
VW	10	10	-	100	100	2.59 ± 0.10

Each treatment contained 5 culture vessels and 15 protocorm units (3-4) were inoculated into each vessel; values are the mean ± SE, derived from observations of 15 seedlings, three randomly selected from each of five culture vessels.

Additionally, the maximum length of the seedlings (2.59 ± 0.10 cm and 2.18 ± 0.12 cm, respectively) was also achieved with these medium combinations (Table 2). Similarly, Utami and Hariyanto (2019) showed improved survivability and seedling development of *Phalaenopsis amboinensis* in VW medium with 15% CW and 10 g/l BH. Whereas, Vilcherrez-Atoche et al. (2020) in the case of *Cattleya maxima* achieved the maximum survival rate, multiplication of PLBs and seedlings differentiation (100%) in MS medium with 30 or 40 g/l banana flour (BF) and 20% CW. On the other hand, Nambiar et al. (2012) found that PLBs growth and proliferation were superior in ½MS medium containing 10 or 20% CW compared to BH or tomato homogenate in the case of *Dendrobium* Alya Pink. In the case of *A. rigida*, Rahman et al. (2013) reported that, 125 ml/l banana extract (BE) supplemented in ½MS medium enhanced protocorm proliferation and seedling growth.

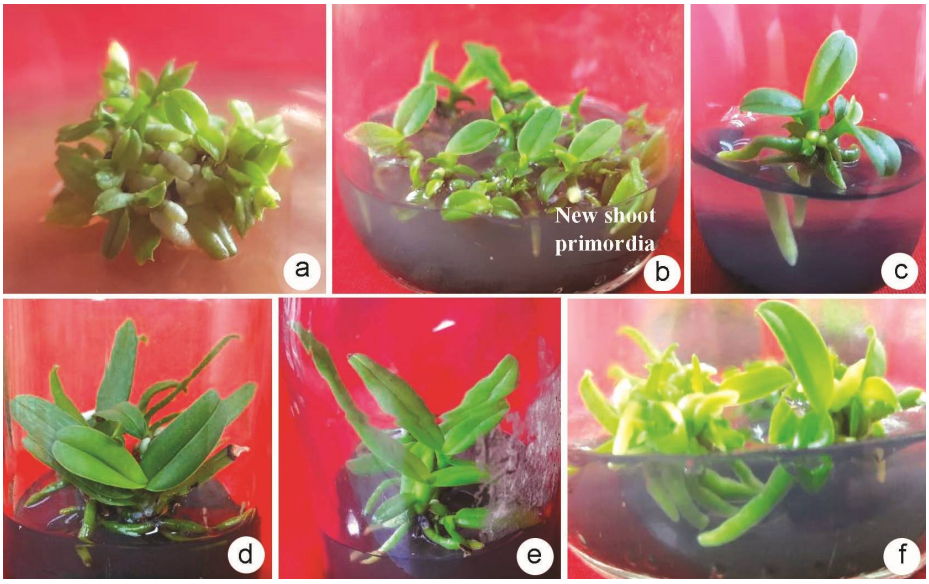

Fig. 2 (a-f). Different stages of seedling development: (a-c) Seedling differentiation in VW + 10% CW + 10 g/l BH after (a) 6 weeks, (b) 8 weeks and, (c) 10 weeks of culture (d-f) Development of *in vitro* germinated seedlings in solidified, (d) VW + 10 g/l BH + 0.1% AC, (e) ½MS + 1.0 mg/l BAP + 0.2 mg/l IBA + 0.1% AC, (f) ½MS + 1.0 mg/l IBA + 0.1% AC.

Regardless of the type of medium used, the addition of activated charcoal (AC) improved the growth and development of the seedlings (Table 3 and 4). However, a hormone-free VW medium containing 10 g/l BH and 0.1% AC yields outstanding results, as measured by shoot number (3.07 ± 0.33); leaf number (4.87 ± 0.26), length (2.11 ± 0.09 cm), and width (0.81 ± 0.04 cm); and root number (3.20 ± 0.31) and length (2.49 ± 0.14 cm) (Table 3 and Fig. 2d). In the same way, Sinha and Roy (2004) in *Vanda teres* produced strong and elongated seedlings and their proliferation by using VW medium with 2.0 g/l banana powder. However, Tantasawat et al. (2015) employed modified VW medium in *Dendrobium* to facilitate the growth and development of seedlings.

Table 3. Effects of BAP, BH and AC on the growth and development of *in vitro* germinated seedlings of *A. rigida* in solidified VW medium, following 10-weeks after inoculation.

Media composition			Number of shoot (Mean ± SE)	Leaf (Mean ± SE)			Root (Mean ± SE)	
BAP (mg/l)	BH (g/l)	AC (%)		Number	Length (cm)	Width (cm)	Number	Length (cm)
1.5	10	-	1.13 ± 0.09	2.27 ± 0.12	0.33 ± 0.03	0.28 ± 0.03	00	00
1.5	-	0.1	1.40 ± 0.16	2.80 ± 0.20	0.58 ± 0.04	0.50 ± 0.04	2.40 ± 0.29	1.53 ± 0.05
-	10	0.1	3.07 ± 0.33	4.87 ± 0.26	2.11 ± 0.09	0.81 ± 0.04	3.20 ± 0.31	2.49 ± 0.14
1.5	10	0.1	2.13 ± 0.24	3.53 ± 0.27	1.80 ± 0.11	0.59 ± 0.05	2.60 ± 0.27	1.84 ± 0.12

Each treatment contained 5 culture vessels, and 15 seedlings were inoculated into each vessel; values are the mean ± SE, derived from observations of 15 seedlings, three randomly selected from each of five culture vessels.

This experiment also investigated the impact of growth regulators and additives on the development of *in vitro* germinated seedlings in ½MS medium (Table 4), however the results were not as promising as with VW (Table 3). Additionally, seedlings grown on ½MS medium containing CW do not show any significant development or root formation.

Table 4. Effects of BAP, banana homogenate, and activated charcoal on the growth and development of *in vitro* germinated seedlings of *A. rigida* in solidified ½MS medium 10-weeks after inoculation.

Media compositions					Number of shoot ($\bar{x}$ ± SE*)	Leaf			Root	
BAP (mg/l)	IBA (mg/l)	CW (%)	BH (g/l)	AC (%)		Number ($\bar{x}$ ± SE*)	Length (cm) ($\bar{x}$ ± SE*)	Width (cm) ($\bar{x}$ ± SE*)	Number ($\bar{x}$ ± SE*)	Length (cm) ($\bar{x}$ ± SE*)
-	-	-	-	-	1.47 ± 0.13	1.73 ± 0.18	0.69 ± 0.05	0.32 ± 0.03	1.67 ± 0.16	0.64 ± 0.05
1.0	-	-	-	0.1	2.87 ± 0.22	3.60 ± 0.25	1.02 ± 0.11	0.43 ± 0.04	1.87 ± 0.19	0.76 ± 0.07
1.5	-	-	-	0.1	2.73 ± 0.21	2.73 ± 0.21	0.91 ± 0.05	0.37 ± 0.02	2.47 ± 0.13	1.60 ± 0.14
1.0	-	-	10	-	1.73 ± 0.21	2.53 ± 0.17	0.41 ± 0.05	0.33 ± 0.03	2.20 ± 0.17	0.85 ± 0.08
-	-	10	10	-	1.00 ± 0.00	2.40 ± 0.13	0.65 ± 0.07	0.31 ± 0.02	00	00
-	-	15	20	-	Although established, seedlings do not exhibit notable growth.					
1.0	-	10	10	0.1	All of the seedlings eventually die.					
1.0	0.2	-	-	0.1	2.93 ± 0.21	3.00 ± 0.20	1.37 ± 0.14	0.45 ± 0.04	2.93 ± 0.21	2.10 ± 0.16
-	1.0	-	-	0.1	2.07 ± 0.27	4.33 ± 0.19	1.30 ± 0.07	0.55 ± 0.03	2.73 ± 0.21	2.43 ± 0.12

Each treatment contained 5 culture vessels, and 15 seedlings were inoculated into each vessel; values are the mean ± SE, derived from observations of 15 seedlings, three randomly selected from each of five culture vessels.

However, the ½MS medium with 1.0 mg/l BAP, 0.2 mg/l IBA and 0.1 % AC produced the most shoots (2.93 ± 0.21) and roots (3.00 ± 0.20) (Table 4 and Fig. 2e), whereas the ½MS medium with 1.0 mg/l IBA and 0.1% AC produced the most leaves (4.33 ± 0.19) with leaf

width and root length (Table 4 and Fig. 2f). In contrast, studies by Poniewozik et al. (2022) on *Paphiopedilum insigne*, Yao et al. (2021) on *P. tigrinum*, and An et al. (2021) on *Sedirea japonica* found that the optimal seedling growth and rooting conditions were achieved using ½MS medium containing 2.0 g/l, 1.0 g/l and 0.6 g/l AC, respectively.

In vitro germinated seedlings of *A. rigida* with 4-5 leaves and 3-4 roots (Fig. 3a) were transplanted to a small clay pot containing coconut husk, tree bark, charcoal and moss with a ratio of 2 : 1 : 1 : 1 for acclimatization (Fig. 3b). Elongation of leaf and root and emergence of new roots and leaves indicated that the seedlings had successfully acclimatized, and the survival rate was more than 80% (Fig. 3c-e). Using the same potting mixture with a ratio of 1 : 1 : 1 : 1, Bhowmik and Rahman (2022) reported 72.38% survival rate in the case of *Eulophia Graminea*. However, Roy (2014) and Suja and Williams (2016) reported over 80% of survival rate in the case of *Acampe praemorsa* after transplanting the seedlings in clay pots consisted of wood charcoal: coco peat and charcoal pieces: brick pieces, respectively in the ratio of 1 : 1.

Fig. 3 (a-e). Acclimatization of seedlings: (a) Well developed seedlings, (b) Acclimatization in small clay pot containing coconut husk, tree bark, charcoal and moss (2 : 1 : 1 : 1), (c-d) 10-weeks old acclimatized hardened plants, (e) Transplanted seedlings on tree bark.

In vitro seed derived propagation technique is a simple approach for multiplication of orchids. This study reports an efficient protocol for large-scale seedling production of *Acampe rigida* through *in vitro* seed germination and seedlings development as well as *ex vitro* acclimatization. ½MS medium supports germination and early development, while VW medium with 10% CW and 10 g/l BH enhances survivability and seedling differentiation. Hormone-free VW with 10 g/l BH and 0.1% AC optimizes complete seedling growth. This *in vitro* regeneration protocol will be helpful for commercial production and conservation of this endangered orchid species, as well as enriching the ornamental and medicinal industries.

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