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In vitro Micropropagation and Mass Multiplication of *Arundina graminifolia* (D. Don) Hochr.- A Terrestrial Medicinal Orchid of Bangladesh

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Key words: *In vitro, Arundina graminifolia*, Medicinal orchid, PGRs, PLBs, MSBs

Abstract

The present study was undertaken to develop an effective protocol for rapid *in vitro* seed germination and mass propagation of *Arundina graminifolia*. Four basal media, *viz.,* MS, B_5, PM and modified VW, were used for germination of seeds and protocorm development. Here, MS medium showed better performance (89.31 ± 0.98%) in seed germination than that of the others. *In vitro* grown plantlets with 2-3 leaves were considered as explants for multiple shoot buds (MSBs) induction and its subsequent development and for that various concentration of PGRs (Kn, BAP and NAA) either single or in combination were used in MS medium. The maximum number of secondary protocorm like bodies (PLBs) (80 ± 0.32%) and shoots (90 ± 0.45%) were recorded on MS medium supplemented with 1.0 mg/l Kn + 1.0 mg/l NAA and 1.0 mg/l BAP respectively. The highest number (14.10 ± 0.21) of secondary PLBs was generated on MS medium in presence of 1.0 mg/l Kn and 1.0 mg/l NAA. Maximum length (4.59 ± 0.14 cm) of plantlets was also obtained on MS medium enriched with 1.5 mg/l BAP and 0.75 mg/l NAA, while a maximum (11.50 ± 0.32) shoots per explant were recorded on the same medium fortified with 1.0 mg/l Kn and 0.5 mg/l NAA. The elongated shoots were transferred on ½MS medium in addition to specific plant growth regulators (PGRs) for root development, where four concentrations (0.5-2.0 mg/l) of PGRs were used. Data showed that the ½MS medium + 1.0 mg/l NAA gave better results on number of roots (9.03 ± 0.26) induction as well as their length (4.92 ± 0.10 cm). Well-rooted plants were transferred to pots after acclimatization. This reproducible protocol can be used for further mass multiplication and applied research in the orchid development in Bangladesh.

Introduction

Orchid is one of the most diverse group of the angiosperm families and are appreciated highly for their long-lasting blossoms, which exhibit a vast range in color, form and scent

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(Kndlmann et al. 2023). They are found all across the world, mainly in the wet tropical areas, but are absent from polar and desert regions (Besi et al. 2023). Mainly, it is cultivated for economic purposes because of its exotic beauty and extended blooming duration (Tiwari et al. 2023). They have aesthetic importance along with medicinal components and ecological value (Choudhary et al. 2023). Their diverse range of specialized pollination and ecological tactics is well-known (Freudenstein and Rasmussen 1999). There are three types of orchids, such as epiphytic, lithophytic and terrestrial, where around 25% of them are terrestrial (De 2020). Several types of orchids grow in Bangladesh naturally in different areas due to its subtropical monsoon climatic conditions (Hossen et al. 2021). *Arundina graminifolia* is a terrestrial perennial plant known as the bamboo orchid and is widely distributed in subtropical and tropical regions of Asia (Martin 2007, Auberon et al. 2016). It is a clumping herb consisting of leafy, erect stems joined at the base, up to 2.5 m tall, and leaves are alternate and grass-like. Flowers are cattleya-like and developed at the tip of the stem. It is used as a folk medicine by indigenous people of different countries, including Bangladesh. It has been widely used for a variety of ethno pharmacological purposes, including rheumatism, food poisoning, snake bites, traumatic injuries, detoxification, and heat relief (Zhang et al. 2021). The ethnic people use it to cure pneumonia, tuberculosis, and bronchitis (Kaur et al. 2022).

Arundina graminifolia (Ai et al. 2019), *Vanda tessellata* (Bhattacharjee et al. 2015a,b), *Rhynchostylis retusa* (Bhattacharjee and Islam 2015), and some other orchids are used in Bangladesh and in China as medicinal plants because of the presence of flavonoids, stilbenoids and phenols in their extracts, which demonstrate antioxidant, antiviral, anticancer and other therapeutic activities. The whole plant is well known as Dai medicine in China and used to treat food poisoning, blood stasis, and liver toxicity (Liu et al. 2017). It is used to treat gastric issues in Malaysia and is known as Ubi bemban (Alsarhan et al. 2014). In India, paste of leaves, stems, and rhizomes is used to cure wounds and cuts, and fresh juice obtained from young leaves and stems is used in the treatment of ear troubles (Singh 2022). In Bangladesh, it is known as Ghasphol (Hossain 2009) and used to treat rheumatism, snake bites, body aches, and bone fractures (Rahman and Huda 2021). In Indonesia, the roots of *A. graminifolia* are used to treat body aches, joint pain, rheumatism, diabetes, tumors, hyperliposis, hepatitis, snake bites and hand and foot fractures (Nurfadilah 2020). The rhizomes of this plant have antibacterial activity (Rahman and Huda 2021).

Orchid seeds are very small and contain undifferentiated embryos that lack enzymes needed to digest polysaccharides and lipids. As a result, orchids require a symbiotic interaction with the mycorrhizal fungus for their successful germination in nature. Knudson (1922) was a pioneer of asymbiotic seed germination of orchids. Later, several others successfully regenerated plantlets through asymbiotic *in vitro* techniques, as reported by Arditti et al. (1981). A large diversity can be seen in the shapes, sizes, and patterns of seeds of Orchidaceae. Seedpods of orchids may contain 1300 to 4 million

seeds (Arditti and Ghani 2000, Bhattacharjee and Islam 2014, Bhattacharjee et al. 2015a). As a member of the orchidaceae family, seed germination of *A. graminifolia* in nature is also rare because of the characteristics of its seeds. Its natural habitat is also shrinking because of overexploitation and climate change, and it is a threatened species of orchidaceae (Verma et al. 2012, Natta et al. 2022). For this reason, seed germination and regeneration of plantlets through *in vitro* techniques are necessary for this medicinally and ornamentally important orchid to survive in nature and further study.

Materials and Methods

Plants of *Arundina graminifolia* were collected from Sylhet, Bangladesh and were maintained in the orchid shed house of the Institute of Biological Sciences (IBSc), University of Rajshahi, under natural conditions. Fresh, immature capsules were collected as the source of seeds and cultured on medium under aseptic conditions.

The capsules were sterilized by washing them under running tap water and later dipping them in an aqueous solution of detergent for 10 min. Then they are dipped in 70% ethanol for 30 sec, followed by treatment with 0.2% $HgCl_2$ solution for 5 min. Finally, the capsules were rinsed several times with double-distilled water before air-drying in a laminar air-flow cabinet. The sterilized capsule was cut with sterilized surgical blades, and the seeds were carefully scooped out and placed on different kinds of sterilized media. Four types of media, *viz.,* MS, PM (P-1056, Phytamax™, Sigma-Aldrich), modified VW (Vacin and Went 1949) and B_5 (Gamborg et al. 1968), were used in this study for seed germination and subsequent protocorm development. MS basal medium was supplemented with 3% sucrose, while PM, B_5 and modified VW were amended with 2% sucrose. The p^H of all media was adjusted to 5.6-5.8. The cultured vessels were kept in the growth chamber for eight hours at 25 ± 2°C under a photoperiod of 16 hrs light and 8 hrs dark cycle. Some of the seeds were removed and spread in one drop of water on a glass slide, where they were viewed under a light microscope. Percentage of germination was calculated by the following formula and repeated five times.

$$\% \text{ of seed germination} = \frac{\text{No. of germinated seeds identified by swelling of embryos} \times 100}{\text{Total no. of seeds.}}$$

The seed germination was first evident by swelling and emergence of the embryo from the testa. Once the spherules (irregularly shaped cell masses) were formed, observations were recorded at an interval of one week to trace different stages of protocorm development. Later, protocorms were taken out aseptically from culture vessels and transferred into fresh culture vessels containing the same germinating medium. Young plantlets with 2-4 leaves and 2-3 weak roots were regenerated from the protocorm. Further culture was done at an interval of 15 days. Before each subculture, the density of seedlings per vessel reduced.

PLBs were collected from the basal end of shoots of *in vitro* grown seedlings of *A. graminifolia* and used as a source of explant for the following experiment. These PLBs were separated and sub-cultured on MS medium supplemented with PGRs either alone or in combination to optimize the cultural conditions for secondary PLBs/shoots formation and subsequent development of secondary PLBs into plantlets. The PGRs used in this study were BAP (0.5, 1.0, 1.5, and 2.0 mg/l), Kn (0.5, 1.0, 1.5, and 2.0 mg/l), and NAA (0.5, 1.0, and 2.0 mg/l). For control, MS basal medium was used. Data were recorded on the basis of the production of several secondary PLBs from a single PLB and their viability to develop plantlets. Percentage of secondary PLBs/shoots formation was recorded by the following formula mentioned below:

$$\% \text{ of secondary PLBs/shoots} = \frac{\text{Responded explants} \times 100}{\text{Total explants}}$$

To increase the number of secondary PLBs, single primary PLBs ware cultured. Increase number of secondary PLBs was counted 30 days after inoculation. Plantlets derived from PLBs were used as explants for other parameters studied in the present experiment.

To evaluate the effect of PGRs on height elongation of plantlets and MSBs induction, they were transferred to MS medium supplemented with different concentrations and combinations of PGRs mentioned previously.

Shoot buds without roots were cultured on half-strength MS medium fortified with auxins for root induction. Different concentrations (0.5, 1.0, 1.5 and 2.0 mg/l) of NAA, IAA, and IBA were used alone for the assessment of their effect on root development. The well-rooted mature plantlets were hardened in different types of substrates (dried moss, sand, soil, brick pieces, cocopeat and charcoal) to assess their efficacy on acclimatization.

Following the parameters, data were recorded on the basis of seed germination, secondary PLBs/shoots, multiplication of secondary PLBs, plant height, and shoot and root development were recorded following the protocol of Bhattacharjee et al. (2025a). Five replicates were used for each parameter. The percentage of secondary PLBs/shoots formation was recorded by the observation of 50 explants for each treatment. Each value represents an average of 30 replicates, and each experiment was repeated three times in case of multiplication of secondary PLBs, plant height development, induction of MSBs, and root development. To investigate the main effect of media states and PGRs and their interaction on micropropagation of *A. graminifolia*, data were assigned to analysis of variance, and the differences of means were separated by the Duncan Multiple Range test (*p-value* at 0.05 levels).

Results and Discussion

Four types of basal media were used to examine their effectiveness on seed germination. Immature capsules were used as a seed source. Seed germination was influenced by the types of medium, and they responded in different ways and at different times. The highest percentage of seed germination was achieved (89.31 ± 0.98) on MS medium with the lowest required time (weeks), followed by PM (73.88 ± 0.95), MVW (56.02 ± 0.82) and B_5 (34.92 ± 1.32) medium (Table 1).

Table 1. Comparative effect of four culture media on seed germination and protocorm development of *A. graminifolia* (D. Don) Hochr.

Culture media	Time required (weeks)		Percentage of seed germination (M ± SE)
	Spherule formation	Protocorm formation	
MS	5 - 6	7 - 8	89.31 ± 0.98[a]
PM	8 - 9	10 - 11	73.88 ± 0.95[b]
Modified VW	10 -11	12 -13	56.02 ± 0.82[c]
B_5	12 -13	14 -15	34.92 ± 1.32[d]

B_5 = Gamborg et al. (1968), MVW (modified medium of Vacin and Went, 1949). MS = Murashige and Skoog (1962), PM = P-1056, Phytamax™, Sigma-Aldrich. Means in a column with the different letter (superscript) are significantly different according to least significant difference at ($p < 0.05$).

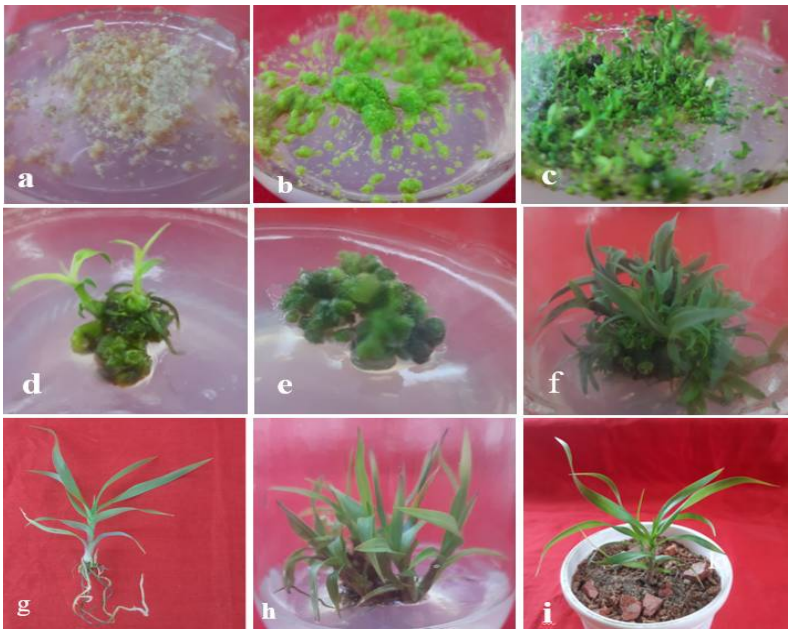

Fig. 1 (a-i). *In vitro* seed germination and secondary PLBs development of *A. graminifolia.* (a) Germinated seeds, (b) Protocorms, (c) Protocorm with shoot apex and leaf primordia, (d) Primary PLBs, (e) Secondary PLBs, (f) Young plantlets developed on secondary PLBs, (g) Elongated plantlet with roots, (h) MSBs, (i) Acclimatized plants transferred to pot.

Within 5-6 weeks, germination was first evident by swelling and emergence of the embryo from the testa. The undifferentiated embryos formed an irregularly shaped cell mass as spherules. Within 1-2 weeks, these spherules turn green and form round-shaped protocorms (Fig. 1a). Protocorms became visible after 7 weeks of culture initiation (Fig. 1b), followed by the formation of vegetative apex and leaf primordia (Fig. 1c). Later, with 2-4 leaves, they developed into young plantlets.

Thirty days old primary PLBs were collected from the basal node of young plantlets (Fig. 1d) and sub-cultured on MS medium supplemented with various forms of auxin and cytokinin to evaluate their efficacy in promoting the development of secondary PLBs/shoots and multiplication of secondary PLBs. Different types of PGRs with different concentrations were used either singly or in combination (Table 2).

Table 2. Effects of PGRs on development of PLBs/shoots and multiplication of secondary protocorm in *A. graminifolia* (D. Don) Hochr.

PGRs (mg/l)			% of secondary PLBs formation (Mean ± SE)	% of shoot formation (Mean ± SE)	No. of secondary PLBs (Mean ± SE)	Required time for PLBs/ shoot formation
BAP	Kn	NAA				
0.5	-	-	-	76 ± 0.51^{b}	-	-
1.0	-	-	-	90 ± 0.45^{a}	-	-
1.5	-	-	24 ± 0.24^{b}	60 ± 0.45^{c}	3.60 ± 0.17^{b}	5-7
2.0	-	-	30 ± 0.32^{a}	50 ± 0.32^{d}	4.50 ± 0.21^{a}	-
-	0.5	-	-	70±0.32^{a}	-	-
-	1.0	-	32 ± 0.37^{b}	58±0.58^{b}	4.80 ± 0.15^{c}	-
-	1.5	-	36 ± 0.24^{b}	50±0.45^{c}	5.40 ± 0.23^{b}	4-6
-	2.0	-	40 ± 0.45^{a}	46±0.51^{d}	6.43 ± 0.19^{a}	-
0.5	-	0.5	-	74 ± 0.32^{a}	-	-
1.0	-	0.5	52 ± 0.37^{bc}	40 ± 0.45^{b}	7.17 ± 018^{c}	-
0.5	-	1.0	44 ± 0.51^{c}	28 ± 0.37^{d}	6.00 ± 0.15^{d}	-
1.0	-	1.0	56 ± 0.40^{b}	30 ± 0.32^{c}	8.40 ± 0.10^{b}	3-5
2.0	-	1.0	60 ± 0.32^{a}	32 ± 0.37^{c}	10.20 ± 0.12^{a}	-
1.0	-	2.0	42 ± 0.37^{c}	26 ± 0.40^{d}	5.00 ± 0.17^{e}	-
2.0	-	2.0	-	-	-	-
-	0.5	0.5	50 ± 0.32^{d}	40 ± 0.45^{a}	4.10 ± 0.15^{f}	-
-	1.0	0.5	60 ± 0.45^{c}	30 ± 0.32^{b}	10.50 ± 0.12^{c}	-
-	0.5	1.0	44 ± 0.60^{e}	26 ± 0.24^{c}	7.87 ± 0.20^{d}	-
-	1.0	1.0	80 ± 0.32^{a}	-	14.10 ± 0.21^{a}	2-3
-	2.0	1.0	70 ± 0.45^{b}	-	12.03 ± 0.15^{b}	-
-	1.0	2.0	56 ± 0.40^{cd}	32 ± 0.20^{b}	6.23 ± 0.18^{e}	-
-	2.0	2.0	-	-	-	-
Control (MSO)			-	20 ± 0.55	-	6-7

PGRs = Plant growth regulators; values represent mean ± SE. Each treatment was repeated three times. Means in a column with different letters (superscript) are significantly different according to the least significant difference at $p < 0.05$ levels.

After 2 weeks of inoculation, primary PLBs started producing secondary PLBs/shoots (Fig. 1e). The maximum percentage of shoot regeneration (90 ± 0.45) occurs on MS medium enriched with 1.0 mg/l BAP, while 1.0 mg/l Kn + 1.0 mg/l NAA supplemented medium provides the highest percentage (80 ± 0.32) of secondary PLBs formation with the lowest required time and also favored the highest multiplication rate (14.10 ± 0.21) of secondary PLBs, followed by 2.0 mg/l Kn and 1.0 mg/l NAA (Table 2). It was observed that the combined effect of Kn and NAA was more effective than other PGRs on the development and multiplication of secondary PLBs of *A. graminifolia,* while the single effect of BAP was proven to be best for shoot formation. Later secondary PLBs were converted successfully into healthy plantlets within 3 weeks (Fig. 1f). Plantlets developed from secondary PLBs were used as explants for further study of plant height elongations, MSBs induction and root development. Some PGRs treated medium did not produce shoots/PLBs. The primary PLBs produced a certain percentage of shoots but failed to develop secondary PLBs in MS basal medium, which was used as a control (Table 2).

Table 3. Effects of MS medium with different PGRs on elongation and multiple shoot buds (MSBs) induction of *A. graminifolia* after 30 days of culture.

PGRs (mg/l)			Elongation of plant height (cm)			No. of MSBs (Mean ± SE)
BAP	Kn	NAA	Final length (Mean)	Initial length (Mean)	Increased length (Mean ± SE)	
0.5	-	-	4.78	3.10	1.68 ± 0.11^{c}	3.33 ± 0.20^{c}
1.0	-	-	5.51	3.19	2.32 ± 0.15^{b}	4.30 ± 0.31^{bc}
1.5	-	-	6.17	3.15	3.02 ± 0.11^{a}	5.70 ± 0.32^{a}
2.0	-	-	5.13	3.12	2.01 ± 0.07^{bc}	4.80 ± 0.35^{ab}
-	0.5	-	4.67	3.20	1.47 ± 0.08^{c}	4.07 ± 0.27^{c}
-	1.0	-	4.93	3.18	1.75 ± 0.10^{c}	5.50 ± 0.29^{b}
-	1.5	-	5.60	3.20	2.40 ± 0.08^{a}	6.83 ± 0.32^{a}
-	2.0	-	5.04	3.15	1.89 ± 0.12^{b}	5.03 ± 0.28^{b}
0.5	-	0.25	5.76	3.20	2.56 ± 0.12^{d}	6.30 ± 0.25^{c}
1.0	-	0.5	6.90	3.17	3.73 ± 0.10^{b}	7.10 ± 0.29^{bc}
1.5	-	0.75	7.77	3.18	4.59 ± 0.14^{a}	8.90 ± 0.26^{a}
2.0	-	1.0	6.33	3.21	3.12 ± 1.15b^{c}	7.80 ± 0.23^{b}
-	0.5	0.25	5.40	3.15	2.25 ± 0.13^{c}	7.00 ± 0.23^{d}
-	1.0	0.5	6.46	3.21	3.25 ± 0.16^{b}	11.50 ± 0.32^{a}
-	1.5	0.75	7.20	3.19	4.01 ± 0.15^{a}	9.93 ± 0.29^{b}
-	2.0	1.0	5.96	3.17	2.79 ± 0.14^{c}	8.80 ± 0.31^{c}
0.5	0.5	-	4.69	3.19	1.50 ± 0.07^{c}	5.13 ± 0.20^{c}
1.0	0.5	-	5.60	3.21	2.39 ± 0.10^{a}	6.60 ± 0.26^{ab}
0.5	1.0	-	5.08	3.18	1.90 ± 0.14^{b}	7.17 ± 0.23^{a}
1.0	1.0	-	4.41	3.20	1.21 ± 0.09^{c}	5.90 ± 0.25^{bc}
Control (MS0)			4.10	3.20	0.90 ± 0.05	2.83 ± 0.19

PGRs = Plant growth regulators, values represent mean ± SE. Each treatment was repeated three times. Means in a column with different letters (superscript) are significantly different according to the least significant difference at $p < 0.05$ levels.

The height elongation of plants and induction of MSBs *in vitro*-grown plantlets were recorded on MS medium fortified with different types of cytokinin and auxins. Different concentrations of BAP (0.5, 1.0, 1.5, and 2.0 mg/l), Kn (0.5, 1.0, 1.5 and 2.0 mg/l) and NAA (0.25, 0.5, 0.75 and 1.0 mg/l) were used either single or in combination to assess their efficacy on above mentioned parameters. Data were recorded after 30 days of inoculation. The highest elongation (4.59 ± 0.14 cm) of plants was observed on MS medium supplemented with 1.5 mg/l BAP and 0.75 mg/l NAA (Fig. 1g and Table 3). Among PGR-fortified mediums, the minimum elongation (1.21 ± 0.09 cm) was obtained from the medium with 1.0 mg/l BAP and 1.0 mg/l Kn; in contrast control medium showed the lowest increased length of height of plantlets, as expected (Table 3).

The maximum number of MSBs (11.50 ± 0.32) per explant was found on MS medium supplemented with 1.0 mg/l Kn and 0.5 mg/l NAA. Among PGR-enriched medium, the minimum number of MSBs (3.33 ± 0.20) per explant was obtained on MS medium fortified with 0.5 mg/l BAP (Fig. 1h**)**. It was observed that the lowest elongation of plants and number of MSBs on MS medium with no PGRs, which were used as a control (Table 3). In both cases data were recorded after 30 days of inoculation.

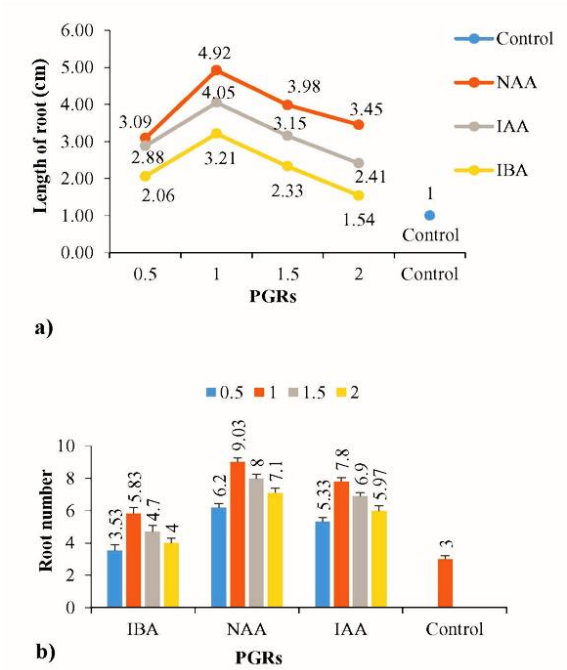

Fig. 2 (a-b). Effects of ½MS medium enriched with auxins on root development of *A. graminifolia*. (a) Length of roots, and (b) Number of roots after 30 days of culture.

Elongated plantlets of *A. graminifolia* were transferred to rooting medium to develop a robust and stout root system. The effect of rooting medium was evaluated by the number of roots and its length developed on plantlets after 30 d of inoculation. To

investigate the mentioned parameters, half-strength MS medium enriched with 0.5, 1.0, 1.5 and 2.0 mg/l concentrations of IAA, NAA, and IBA was used as a rooting medium (Fig. 2a, b). The maximum number of roots (9.03 ± 0.26) per shoot and highest length (4.92 ± 0.10) were achieved on MS medium fortified with 1.0 mg/l NAA. In the case of auxin-supplemented media, IAA also proved to be effective for root induction. The table data shows that IBA was less effective for root induction but the lowest root induction was observed on MS basal medium, which was used as a control. We noticed a significant difference in root formation and its length at different concentrations of auxins (Fig. 2a, b). Elongated plantlets with well-developed roots were acclimatized in pots (Fig. 1i). Different types of substrates were used for the hardening of *in vitro* grown plantlets of *A. graminifolia*. We obtained the highest surviving plants (85%) with increased length (3.20 ± 0.06) when we acclimatized them in pots containing dried moss, coco peat, and brick pieces at a 1 : 1 : 1 ratio (Table 4). Later, we shifted them to the natural conditions of field habitats.

Table 4. Effects of different substrates on survival rate and plant height elongation of *Arundina graminifolia* during acclimatization.

Substrate used for hardening	Survival (%)	Increased plant height (cm) Mean ± SE
Dried moss + cocopeat + brick pieces (1 : 1 : 1)	85	3.20 ± 0.06[a]
Dried moss + brick pieces + soil (1 : 1 : 1)	70	2.76 ± 0.03[ab]
Brick pieces + cocopeat + charcoal (1 : 1 : 1)	65	2.30 ± 0.03[b]
Dried moss + sand + soil (1 : 1 : 1)	50	2.10 ± 0.04[c]

Means in a column with the different letter (superscript) are significantly different according to least significant difference at ($p < 0.05$).

Data on the percentage of survived plants were recorded by the observation of twenty plants for each treatment, and increased plant height was recorded by the observation of ten replicates. Data were recorded after 30 days of initiation of acclimatization.

This is the first complete protocol for mass multiplication of *A. graminifolia* by PLBs-derived plantlets in Bangladesh. The nutrient requirements for orchid seed germination are assumed to be species-specific. Different plant tissue culture media have been adapted for orchid seed germination (Nadarajan et al. 2011, Bhattacharjee and Islam 2014, Islam and Bhattacharjee 2015, Ayesha and Islam 2024). In the present study, four types of basal media were used to investigate its effectiveness on *in vitro* seed germination and protocorm formation of *A. graminifolia*. Results showed that MS media promoted seed germination of *A. graminifolia* better than other media. We obtained 89.31% seed germination on MS media, which was the highest among all media used in this study. The present findings are in agreement with the result of terrestrial orchid species *Erythrodes humilis* (Bhowmik and Rahman 2020a). Devi (2021) also reported MS as

the best medium for seed germination of *A. graminifolia*. In contrast, Bhadra and Bhowmik (2005) reported PM medium was more effective than MS medium for seed germination of *A. graminifolia.* Lalduhsanga et al. (2021) reported that ½MS semi-solid medium was best for seed germination, and its liquid form was best for protocorm development of *A. graminifolia*. The present findings also supported the results of several epiphytic orchid species, such as *Dendrobium* and other orchids (Barman et al. 2020, Ayesha and Islam 2024). On the contrary, PM medium provided the highest seed germination percentage in case of a terrestrial orchid, *S. plicata* (Bhowmik and Rahman 2020b). Furthermore, MS medium supplemented with PGRs was proved to be best for seed germination, protocorm development, and PLBs formation of *Phaius tankarvilleae* and *Vanda* sp. (Pant et al. 2011, Bhattacharjee et al. 2015a).

Development of PLBs is very important for *in vitro* micropropagation of orchids (Islam et al. 2015). The present study showed the successful development of secondary PLBs/shoots from primary PLBs. MS medium enriched with PGRs seems to have an effect on the response of secondary PLBs/shoots formation. In the present study, MS medium enriched with 1.0 mg/l Kn + 1.0 mg/l NAA provides the highest percentage of secondary PLBs formation, while 1.0 mg/l BAP provides the highest percentage of shoot formation. The maximum number of secondary PLBs also developed in medium supplemented with 1.0 mg/l Kn + 1.0 mg/l NAA. Alam et al. (2020) noted the beneficial effect of alginate-treated MS medium on PLBs formation in *Dendrobium kngianum* Bidwill ex Lindl. Ona and Shimasaki (2023) reported MS medium enriched with citric acid (CA) significantly increased the percentage of PLBs and shoot formation in *Cymbidium floribundum.* Several publications showed that the combination of cytokinin and auxin is beneficial for PLBs development (Bhattacharjee and Islam 2015, Hossen et al. 2021). On the contrary, Martin (2007) reported that ½MS medium with BAP facilitated the formation of PLBs from the nodal segment of field-grown *A. graminifolia*.

The height elongation of plantlets and the induction of MSBs are two major steps of *in vitro* micropropagation. The present findings showed that the optimum rate of elongation of plantlets was recorded on MS medium with 1.5 mg/l BAP and 0.75 mg/l NAA. Here, results are partially supported by the findings of another terrestrial orchid, *Geodorum densiflorum* (Bhadra and Hossain 2003). Several investigators have identified beneficial effects of cytokinin combined with auxin on height increase of plantlets of some orchids, such as *Cymbidium finlaysonianum* and *Erythrodes humilis* (Islam et al. 2015, Bhowmik and Rahman 2020a). Under this study, 1.0 mg/l Kn combined with 0.5 mg/l NAA induced the highest rate of MSBs induction. This outcome matches up with the results of research on *Phaius tankervilleae* carried out by Thokchom et al. (2017). Some researchers reported that the positive effect of MS medium with BAP on multiple shoot bud induction of terrestrial orchid species*, Phaius tankervilleae,* and *Eulophia dabia* (Pant and Shrestha 2011, Panwar et al. 2022). On the contrary, Gegi et al. (2018) reported the combined effect of Kn and BAP was beneficial on multiple shoot induction of the terrestrial orchid *Geodorum densiflorum.* In terms of both root production and length,

NAA was significantly better compared to other auxins employed in this study. The beneficial effect of NAA on root induction of *Geodorum densiflorum, Malaxis acuminata,* and *Phaius tankervilleae* was also reported by Sheelavantmath et al. (2000), Pant and Shrestha (2011) and Thokchom et al. (2017). On the contrary, some researchers reported that the very positive effect of MS medium with IBA on root induction of the terrestrial orchid *Erythrodes humilis* (Bhowmik and Rahman 2020a). On the other hand, several researchers reported on the superior effect of IAA on root development of many epiphytic orchid species. For acclimatization, the well-rooted plantlets were transferred from culture vessels to pots with different types of substrates. Data showed that 85% survived plants with a 3.20 cm increased length, which was highest when acclimatized in pots containing dried moss, cocopeat, and brick pieces at a 1 : 1 : 1 ratio. After 2 weeks, they established it under natural conditions at the IBSc shade house in the research field under Rajshahi University.

The present study describes a complete suitable approach for *in vitro* asymbiotic seed germination and mass multiplication of *Arundina graminifolia*. In this case, the MS medium was ideal for seed germination and protocorm development. PGRs combinations were more productive for subsequent development of PLBs, MSBs and plant height. NAA was found to be efficient for root induction. *In vitro* grown mature plantlets survived better in dried moss, cocopeat, and sand at a ratio of 1 : 1 : 1.

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