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Research Paper

Optimization of Nutrient Requirements for Asymbiotic Seed Germination of *Dendrobium longicornu* Lindl. and *D. formosum* Roxb.

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High frequency plantlet regeneration via seeds of *Dendrobium longicornu* Lindl. and *D. formosum* Roxb. was tried in four different media namely, Murashige and Skoog (MS), Gamborg *et al.*, (B_5), Mitra *et al.*, and Knudson C (KC). The embryos emerged out of the swollen seeds within 3-5 weeks of culture and developed into well-defined yellowish-green protocorms. The optimum germination was recorded on MS medium for both the species wherein 90-95% and 80-85% seed germination was recorded for *D. longicornu* and *D. formosum* respectively. The development of seedlings (namely, shoot number, shoot length, root number and root length) was also enhanced on MS medium as compared to other media tried. Incorporation of growth regulators viz., IAA (0.057, 0.57, 5.70 and 28.54 μM), BAP (0.044, 0.44, 4.4 and 22.49 μM) and GA_3 (0.028, 0.28, 2.88 and 14.43 μM) individually in the medium affected the germination response. In case of D. longicornu, the optimal response was recorded in medium containing 0.057 μM IAA, whereas seed germination and protocorm size improved in *D. formosum* with the incorporation of 0.44 μM BAP in the medium. Well developed plantlets obtained after 20-25 weeks of culture were transferred to thermocol pots and the highest survivability of transferred plantlets (68%) in case of *D. longicornu*, was recorded in substratum containing brick pieces, charcoal chunks and bark pieces (1:1:1) with a top layer of moss. Around 87% of *D. formosum* transferred plantlets survived in substratum containing brick pieces, charcoal chunks and decaying litter (1:1:1) with a layer of moss. The results obtained in the present study shows that the nutrient requirements of both the dendrobes are specific for asymbiotic seed germination.

Key Words: Dendrobium spp.; Orchid seeds; Asymbiotic germination; Over-exploitation; Endangered species

Introduction

Orchids are well represented in Northeast India (about 750-800 species) [1]. Over-exploitation and habitat destruction caused by increasing human population and natural calamites have created serious threat to the plant diversity of the region. Moreover, the commercial exploitation of ornamental orchids has considerably depleted their natural populations in the wild. Presently, all the orchids have been included under endangered species of plants [2]. To avoid this serious threat, attempts should be made to multiply the orchids so as to prevent them from extinction. In orchids, the embryo is few-celled at the time of seed maturation and its proper development takes place only during germination of seeds [3]. Moreover, the orchid seeds do not have sufficient reserve food materials to take care of the growth of embryo during germination [4]. These seeds have to depend on some external source for nutrients so as to enable their undifferentiated embryo to germinate and develop into a protocorm. Mycorrhizal association with particular fungal species is necessary for early stages of development because of which less than 5% of the orchid seeds germinate in nature [5]. The *in vitro* seed germination has an added advantage to overcome this reproductive barrier as well as for the conservation of the orchid species. Although *in vitro* asymbiotic seed germination has been achieved in many orchids [6-11], the reports so far depict that the responses of orchids vary from species to species and a thorough study on each and every species of orchid is required.

In this paper we report the nutrient requirements for asymbiotic seed germination of two ornamentally important dendrobes which have long-lasting flowers that contribute significantly towards the ornamental industry. *Dendrobium longicornu* Lindl., has been reported to be an endemic species in North-east India [1] (Fig. 1a). The species is also medicinally important [12] and as such has been included in CITES [13]. Dendrobium formosum Roxb., is a rare and endangered orchid of Northeast India [14] and has one of the largest flowers amongst the dendrobes (Fig. 2a). This species of dendrobes also forms hybrids of outstanding merit.

Material and Methods

Plants of *D. longicornu* and *D. formosum*, collected from the forests of Meghalaya, were maintained in the glass house of the Plant Biotechnology Laboratory, Centre for Advanced Studies in Botany, North Eastern Hill University, Shillong. To obtain sufficient number of capsules the flowers were hand pollinated. About eight-

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months old capsules were harvested from the plants and washed thoroughly under running tap water. The capsules were surface sterilized by dipping in 70% ethyl alcohol for about 3 min followed by flaming. This process of flaming and sterilization was repeated 2-3 times and then the capsules were split open aseptically, seeds scooped out and inoculated on four different media, *viz.*, Murashige and Skoog (MS) [15], Knudson C (KC) [16], Mitra *et al.* (Mitra) [17], and Gamborg (B_5) [18]. The media were incorporated with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The pH of the media was adjusted to 5.8 ± 0.02 using 1N NaOH prior to autoclaving at 121°C for 15 min. Each treatment had 10 replicates and the experiments were repeated twice. Different growth regulators with varying concentrations viz., Indole-3-acetic acid (IAA) at 0.057, 0.57, 5.70, and 28.54 μM, 6-Benzyl adenine (BAP) at 0.044, 0.44, 4.4 and 22.49 μM and Gibberrellic Acid (GA_3) at 0.028, 0.28, 2.88 and 14.43 μM were also added singly in the optimal medium. The cultures were incubated at 25 ± 2°C for 12h photoperiod under 50 μmol $m^{-2}s^{-1}$ light intensity. The protocorm volume was determined using the formula for an oblete speroid $4/3\ \pi\ a^2\ b$, where a and b are minor and major semi-axes respectively [7]. For further studies on seedling growth and development in different basal media *viz.*, MS, B_5, Mitra and KC, 3 months old seedlings measuring 0.5 – 0.7 cm in size (bearing two leaf initials without any root) were cultured. Observations of seedlings for growth parameters (shoot number and length, and root number and length) were made after 90 days of culture. Five replicates were taken from each treatment and the experiments repeated twice. The data was subjected to statistical analysis using one way ANOVA and comparisons between the mean values of treatments were made by Fisher's LSD test) [19]. The seedlings with well defined roots (2-3 cm in length) were transplanted into clean thermocol pots of 8 cm diam. containing different mixtures of composts *viz.*, (i) brick pieces and charcoal chunks (1:1), (ii) brick pieces and charcoal chunks (1:1) + a top layer of moss, (iii) brick, charcoal chunks and decaying litter (1:1:1), (iv) brick pieces, charcoal chunks and decaying litter (1:1:1) + a top layer of moss, (v) brick pieces, charcoal and bark pieces (1:1:1), (vi) brick pieces, charcoal chunks and bark pieces (1:1:1) + a top layer of moss. The survivality of the transferred plantlets was recorded after 90 d of transfer.

Results and Discussion

Initiation of seed germination and seedling development varied with the medium employed for both the dendrobes studied. The maximum seed germination for both *D. longicornu* (95.55 %) and *D. formosum* (84.90 %) was recorded in MS medium (Table 1, 2; Fig. 1b-e, Fig. 2b-e). The development of seedlings in terms of shoot number and length, and root number and root length, was also found to be the best in MS medium as compared to the other media tried (Table 3, 4; Fig. 1f-i, Fig. 2f-i). The suitability of MS medium for seed germination and seedling development of both the dendrobes could be attributed to the fact that MS medium is rich in both macro- and micro nutrients. The appropriate source of nitrogen is the essential feature of a balanced medium. Kramer and Kozlowski reported that nitrogen present in MS medium greatly influences the growth and differentiation of cells [20]. Ammonium content was highest in MS medium, and may have contributed to the highest overall germination rates. It is

Table 1. Effect of different media on seed germination and size of protocorms of *D. longicornu*

Medium	Germination (%)*	Time taken for protocorm formation (in weeks)	Volume (mm^3)*
MS	95.55 ± 0.52^a	3-4	0.171 ± 0.009^a
B_5	88.64 ± 0.57^b	3-4	0.100 ± 0.008^b
Mitra	83.14 ± 0.43^c	4-5	0.072 ± 0.005^c
KC	77.42 ± 1.01^d	4-5	0.067 ± 0.005^c

Values are mean ± S.E. Means followed by same letter in the column are not significantly different as indicated by Fisher's LSD (p = 0.05)
* Data recorded after 2 months

Table 2. Effect of different media on seed germination and size of protocorms of *D. formosum*

Medium	Germination (%)*	Time taken for protocorm formation (in weeks)	Volume (mm^3)*
MS	84.90 ± 0.55^a	3-4	0.57 ± 0.07^a
B_5	74.98 ± 1.00^b	3-4	0.29 ± 0.04^b
Mitra	69.87 ± 0.51^c	3-4	0.14 ± 0.004^c
KC	57.88 ± 0.64^d	3-4	0.13 ± 0.01^c

Values are mean ± S.E. Means followed by same letter in the column are not significantly different as indicated by Fisher's LSD (p = 0.05)
* Data recorded after 2 months

Table 3. Effect of different media on growth and development of *D. longicornu* seedlings after 90 days of culture

Media	Shoot no. *	Shoot length (cm)	Root no. *	Root length (cm)
MS	2.2±0.06^a	2.3±0.04^a	3.7±0.28^{ab}	2.63±0.12^a
B_5	1.25±0.04^b	1.4±0.04^b	2.9±0.26^{bc}	1.92±0.02^b
Mitra	1.25±0.07^b	1.45±0.06^b	2.65±0.18^c	1.80±0.07^b

Values are mean ± S.E. Means followed by same letter in the column are not significantly different as indicated by Fisher's LSD (p = 0.05)

Table 4. Effect of different media on growth and development of *D. formosum* seedlings after 90 days of culture

Media	Shoot no. *	Shoot length (cm)	Root no. *	Root length (cm)
MS	2.42±0.08^a	2.50±0.20^a	3.35±0.51^a	1.45±0.006^a
B_5	1.50±0.05^b	1.12±0.04^b	3.10±0.21^a	1.17±0.09^b
Mitra	1.45±0.09^b	1.02±0.02^b	2.50±0.05^a	1.10±0.04^b

Values are mean ± S.E. Means followed by same letter in the column are not significantly different as indicated by Fisher's LSD (p = 0.05).

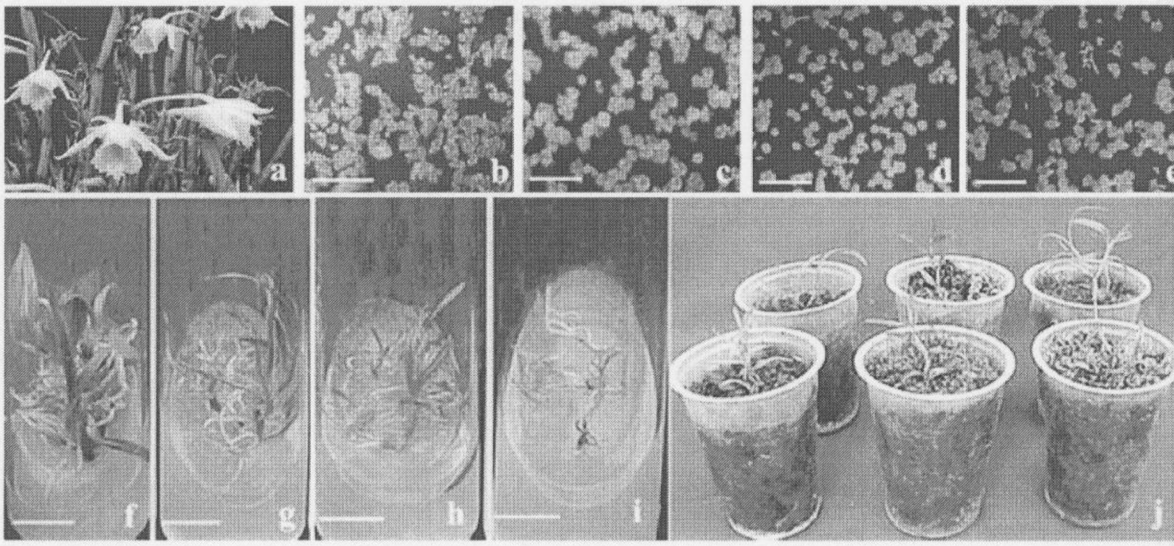

Fig. 1: *Dendrobium longicornu* blooming (**a**), asymbiotic seed germination after 2 months on MS (**b**), B_5 (**c**), Mitra (**d**) and KC (**e**) media, seedling growth after 3 months of culture in MS (**f**), Mitra (**g**), B_5 (**h**) and KC (**i**) media, hardened plants (**j**).
(b-e) *bar = 1 mm,* ***(f-i)*** *bar = 1 cm*

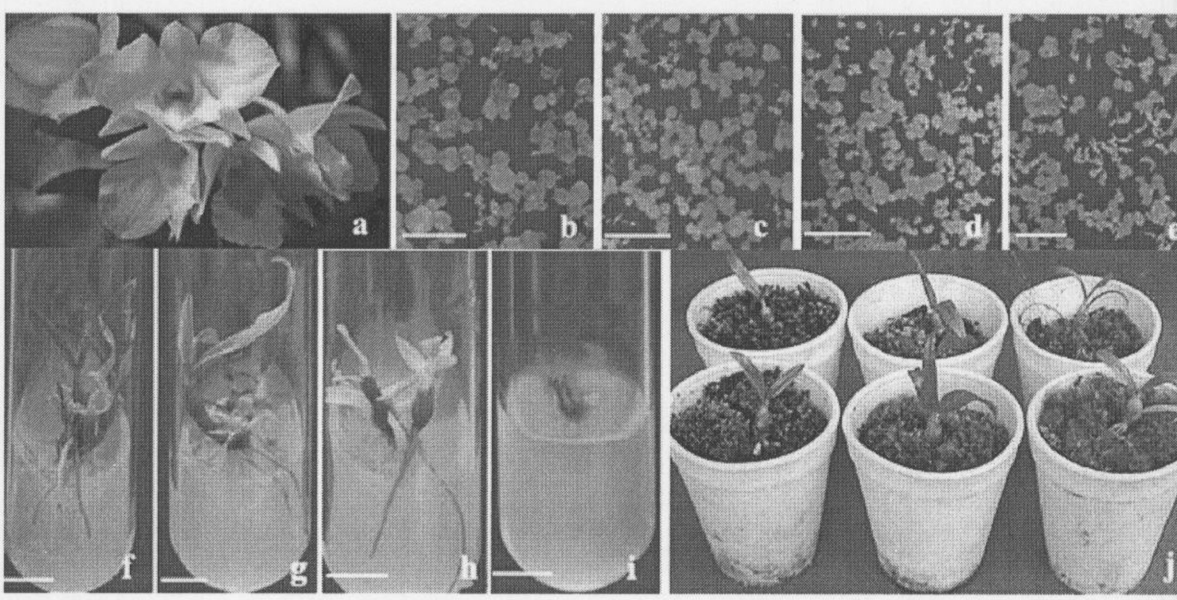

Fig. 2: *Dendrobium formosum* blooming (**a**), asymbiotic seed germination after 2 months on MS (**b**), Mitra (**c**), B_5 (**d**) and KC (**e**) media, seedling growth after 3 months of culture in MS (**f**), Mitra (**g**), B_5 (**h**) and KC (**i**) media, hardened plants (**j**). ***(b-e)*** *bar = 1 mm,* ***(f-i)*** *bar = 1 cm*

well known that the rapid assimilation and use of NH_4^+– N in most plants cause reduced inhibition of germination as compared to NO_3^- – N which accumulates in toxic quantities) [21]. The presence of nitrogen in the form of ammonium nitrate in MS medium could have been the most suitable source for seed germination and seedling development of *D. longicornu* and *D. formosum* (Table 5). The presence of NH_4^+ in the form of ammonium sulphate in Mitra, B_5 and KC and NO_3^- in the form of calcium nitrate in Mitra and KC may have had inhibitory influence on seed germination and seedling growth of both the dendrobes. Although there are reports of enhanced seed germination of orchids in KC medium [6, 22-24], in the present study it was found that seeds of *D. longicornu* and *D. formosum* responded poorly to KC medium and growth of seedlings was completely inhibited in it. This is in agreement to the some of the earlier reports wherein seed germination of some epiphytic species was inhibited in KC medium [25,26].

The incorporation of growth regulators in the medium did not have significant effects on seed germination and development of *D. longicornu* and *D. formosum* (Tables 6 & 7). The optimum seed germination with increased size of the protocorms for *D. longicornu,* was observed in medium supplemented with 0.057μM of IAA, whereas in case of *D. formosum* the germination as well as the size of the protocorms was enhanced at 0.44 μM BAP in the medium. The enrichment of nutrient media with growth regulators have been widely used for orchid seed germination. Both the inhibitory [27] and promotory [28,29] effects of IAA in the medium were observed in several orchids. Although the enhanced effect of BAP was also observed in some orchids [30,31], an exogenous supply of BAP at lower concentrations (0.44 -0.88 μM) was reported to be critical for germination of *Cypripedium* species [32-34]. In the present study, the maximum seed germination in both *D. longicornu* and *D. formosum* was observed in medium supplemented with 0.44 μM BAP, and with increase in the concentration of BAP inhibited/reduced germination resulted. The incorporation of GA_3 in the medium totally inhibited the germination of seeds in both the orchid species studied. Earlier, Kano (1965) had also observed inhibitory effects of GA_3 on some of the species of *Bletilla*, *Brassolaeliocattleya* and *Dendrobium* [35].

Table 5. Inorganic, organic components and gelling agent of the media

Costituents	MS	Mitra	B_5	KC
Inorganic salts (mg/l)				
NH_4NO_3	1650.00	–	–	–
KNO_3	1900.00	180.00	2500.00	180.00
KH_2PO_3	170.00	–	–	150.00
$Ca(NO_3)_2.4H_2O$	–	200.00	–	200.00
$MgSO_4.7H_2O$	370.00	200.00	250.00	250.00
$(NH_4)_2SO_4$	–	100.00	134.00	100.00
KI	0.83	0.03	0.75	80.00
H_3BO_3	6.20	0.06	3.00	6.20
$MnSO_4.4H_2O$	22.30	0.40	10.00	0.075
$ZnSO_4.7H_2O$	8.60	0.05	2.00	–
$Na_2MoO_4.2H_2O$	0.25	0.05	0.25	0.25
$CuSO_4.5H_2O$	0.25	0.05	0.025	0.025
$CoCl_2.6H_2O$	0.025	–	0.025	0.025
$FeSO_4.7H_2O$	27.80	27.80	27.80	27.30
$Na_2EDTA.2H_2O$	37.30	37.30	37.30	37.30
$CaCl_2.2H_2O$	440.00	–	150.00	–
$CO(NO_3)_2.6H_2O$	–	0.05	–	–
$NaH_2PO_4.2H_2O$	–	250.00	150.00	–
$MnCl_2$	–	–	–	3.90
Organic nutrients (mg/l)				
Thiamine HCl	0.10	0.30	10.00	0.30
Nicotinic Acid	0.50	1.25	–	–
Pyridoxine HCl	0.50	0.30	–	0.30
Glycine	2.00	–	2.00	–
Riboflavin	–	0.05	–	0.30
Biotin	–	0.50	–	–
Folic Acid	–	0.30	–	–
Meso Inositol	100.00	–	100.00	–
Sucrose	30000.00	20000.00	20000.00	20000.00
Gelling agents (g/l)				
Agar	8.00	9.00	8.00	12.0
pH	5.8	5.6	5.8	5.2

Table 6. Effect of IAA and BAP on seed germination and size of protocorms of *D. longicornu*

Growth regulators	Concentration (μM/l)	Germination (%)*	Time taken for protocorm formation (in weeks)	Volume (mm^3)*
	0.057	94.44 ± 0.45^a	3-4	0.293 ±0.039^a
	0.57	89.83 ± 0.57^b	3-4	0.151 ±0.023^b
IAA	5.70	82.87 ± 0.45^{cc}	3-4	0.087 ±0.008^b
	28.54	–	–	–
	0.044	81.34 ± 0.55^d	4-5	0.093 ±0.012^b
BAP	0.44	81.71 ± 0.42^{cd}	4-5	0.096 ±0.009^b
	4.4	–	–	–
	22.49	–	–	–

Values are mean ± S.E. Means followed by same letter in the column are not significantly different as indicated by Fisher's LSD (p = 0.05)
* Data recorded after 2 months.

Table 7. Effect of IAA and BAP on seed germination and size of protocorms of *D. formosum*

Growth regulators	Concentration (μM/l)	Germination (%)*	Time taken for protocorm formation (in weeks)	Volume (mm^3)*
	0.057	71.71 ± 0.58^c	3-4	0.225± 0.019^{dc}
IAA	0.57	67.41 ± 1.44^d	3-4	0.176 ± 0.009^e
	5.70	–	–	–
	28.54	–	–	–
	0.044	81.29 ± 0.54^b	3-4	0.634 ± 0.07^b
BAP	0.44	91.78 ± 0.50^a	3-4	0.963 ± 0.08^a
	4.4	64.87 ± 2.45^d	3-4	0.370 ± 0.03^{cd}
	22.49	–	–	–

Values are mean ± S.E. Means followed by same letter in the column are not significantly different as indicated by Fisher's LSD (p = 0.05)
* Data recorded after 2 months

According to Arditti and Ernst (1984), orchid seedlings are capable of synthesizing their requirements of gibberellins and have a limited ability to deactivate the hormone. The exogenous supply of gibberellins usually proves detrimental to their growth [36].

The plantlets obtained after 20-25 weeks of culture were acclimatized successfully in pots, where the highest survivability of 68% was obtained in case of *D. longicornu* in substratum containing brick pieces, charcoal chunks and bark pieces (1:1:1) with a layer of moss, while a survivability of 87% in *D. formosum* was obtained in substratum containing brick pieces, charcoal chunks and decaying litter (1:1:1) with a layer of moss (Fig. 1j, Fig. 2j). The survivability of the micropropagated plantlets on being transferred to pots depends on proper acclimatization of plantlets. Both these two dendrobes being epiphytic in nature need a good growing substratum having properties such as maximum water holding capacity, porosity and drainage for proper growth and development of *in vitro* raised plants. Charcoal and brick pieces used as a substrate provide good drainage and adequate aeration to the roots, which is of primordial importance to the orchids. A layer of moss on top proved to be beneficial for retaining the moisture content at a higher level.

Considering the commercial and threatened status of these orchid species in nature, the present study provides simple protocols for large scale regeneration, establishment and conservation of *Dendrobium longicornu* and *D. formosum* through asymbiotic seed germination.

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