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Kuntala N Barua
Rain Forest Research Institute, Sotai, Jorhat, Assam, India

Bebija L Singha
Rain Forest Research Institute, Sotai, Jorhat, Assam, India

Satyam Bordoloi
Rain Forest Research Institute, Sotai, Jorhat, Assam, India

Bonanya Bora
Rain Forest Research Institute, Sotai, Jorhat, Assam, India

Corresponding Author:
Kuntala N Barua
Rain Forest Research Institute, Sotai, Jorhat, Assam, India

In vitro seed propagation and mass multiplication of some magnificent Orchids of Northeast India

Kuntala N Barua, Bebija L Singha, Satyam Bordoloi and Bonanya Bora

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Abstract
Eastern Himalaya owing to its specific gradient and varied climatic conditions has cradled the wonderful natural heritage of temperate and sub-tropical orchids. Large scale forest conversion and over exploitation has brought the orchid diversity to the brink of extinction. Increasing vulnerability of these species it is an urgent need for conservation. *In vitro* seed propagation is the popular multiplication technique and indispensable components for conservation and restoration of rare and endangered orchids. Study highlighted *In vitro* seed propagation of ten magnificent orchid species of Northeast India *viz. Aerides multiflora, Cleisocentron pallens, Cymbidium aloifolium, Dendrobium aduncum, Dendrobium fimbriatum, Dendrobium lituiflorum, Dendrobium moschatum, Phalaenopsis mannii, Phaius tankerville* and *Rhynchostylis retusa.* MS medium exihibited better proliferation of seeds followed by Mitra *et al*. medium. Lower amount of BAP was most effective for transforming the spherules into fully developed protocorm like bodies. Study revealed that, culture media supplemented with 1.5, mgL^{-1} BAP recorded maximum number of shoot (3.9±0.88) with length of 5.56±0.58 cm. for *A. multiflora.* Highest number (11.2±1.75, 4.1±0.74) and elongation of shoot (4.75±0.58 cm, 5.13±0.85 cm) were recorded in 1.5 mgL^{-1} BAP and 0.5 mgL^{-1} IBA after 8 week of the culture for *C. pallens* and *R. retusa* respectively. However, media supplemented with 0.2% activated charcoal Powder, 0.2% potato extract, 0.2% yeast extract and 0.2% banana extract as natural additives showed excellent performance. MS medium supplemented with TDZ (1.5 mgL^{-1}) was best phytohormons for shoot elongation of *D. moschatum*. Root establishment of majority of species found high when medium supplemented by IBA (1.5 mgL^{-1}). Highest survivality of seedlings established in potting medium containing Brick chips: Charcoal: Sphagnum moss (1:1:2).

Keywords: *In vitro* seed propagation, mass multiplication, conservation, Orchids, Northeast India

Introduction
Orchids are among the most advanced flowering plants showing incredible diversity in form and structure, belonging to the monocotyledon family *Orchidaceae*. Eastern Himalaya including Assam owing to its specific gradient and varied climatic conditions has cradled the rich heritage of temperate and sub-tropical orchids accounting for about 828 species. The process of ruthless destruction, large scale conversion of forest and over exploitation has brought some of the orchid diversity to the brink of extinction and a large number of them have been rendered vulnerable. Fruit setting of the orchid species is also exceptionally fewer. Hence, the orchid family is in the position of rarity and considered under the APPENDIX-I&II of the CITES (Convention on the International Trade in Endangered Species of Fauna and Flora). Earlier Vij (2001) [1] stated that Indian sub-continent has been a desired orchid hunting ground that augmented destruction of specific habitats and some species vanished from the Indian regions. Increasing vulnerability of these species it is an urgent need to restore the ecology of their habitat for effective conservation along with mass multiplication.

In vitro seed propagation is the most popular multiplication technique and essential components of genetic resource management, conservation and restoration of rare and endangered species (Fay 1992) [2]. Present study was carry out *In vitro* seed propagation of ten magnificent orchid species of Northeast India namely *Aerides multiflora, Cleisocentron pallens, Cymbidium aloifolium, Dendrobium aduncum, Dendrobium fimbriatum, Dendrobium lituiflorum, Dendrobium moschatum, Phalaenopsis mannii, Phaius tankerville* and *Rhynchostylis retusa. Dendrobium*s are prized for floricultural excellence. Similarly, *Aerides, Phaius* and *Rhynchostylis* have fascinated the commercial growers for floriculture business

because of their attractive and long-lasting flower. *Cleisocentron pallens* and *Phalaenopsis mannii* are two spectacular monopodium orchids gradually depleted in the forest of Northeast India. However, work on *In vitro* seed propagation was still inadequate (Soetopo and Pumamaningsih, 2012, Nongdam and Tikendra, 2014) [3, 4]. Therefore, *In vitro* seed propagation technique was adopted for successful multiplication and conservation approach.

Materials and Methods

Collection of capsule and surface sterilization

Fully mature capsules were collected from the Forest of North Eastern Coal Field under Dihing-Patkai WLS (95° 36′ 12" E to 96° 01′ 55" E and 27° 12′ 20" N to 27° 26′ 27" N) during the month of December-January 2019. Capsules were washed properly in Tween-20 solution for 10 minutes (2-3 drops in 100ml sterile distilled water), dripped in 70% ethanol for 60 seconds, followed by surface sterilized using 0.5% $HgCl_2$ solution for 10 minutes. The capsules were washed by sterile distilled water for complete remove of $HgCl_2$ and go after by flaming. The sterilised capsules were split open longitudinally by using sterile surgical blade to scooped out minute seeds and spread out on the culture media. The whole procedure was performed in aseptic condition under laminar flow to prevent any kind of contaminations.

In vitro seed culture medium

The mature seeds of each species were inoculated on different seed germination media (full-strength of MS medium, half-strength of MS, IY and Mitra *et al.*). The media were solidified by using 0.7% agar and P^H adjusted to 5.8 with 1N NaOH or 1N HCl, autoclaved at 121°C for 15 minutes. The cultures were maintained at 25°C±2°C with proper light illumination under 16/24 h photoperiod and germination percentages were recorded.

In vitro shoot formation and regeneration

The protocorms were scooped and culture in MS and Mitra *et al*. media addition with various plant growth regulators [6-Benzyl Amino Purine (0.5-2 mgL^{-1}) and Indole-3-butyric Acid (0.5-2 mgL^{-1})], either singly or in combination. Yeast extract, banana extract, potato extract and activated charcoal powder were used as natural additives.

In vitro root formation

Individual 5-6 cm long shoot were selected for root initiation and development that transferred to MS medium supplemented with different concentration of Indole-3-butyric Acid (0.5-2 mgL^{-1}) and 1-Naphthalene Acetic Acid (0.5-2 mgL^{-1}).

Acclimatization of seedlings

Rooted seedlings were taken out from culture vessels by using sterile forceps, thoroughly washed with distilled water to remove the gel adhered and treated with 0.1% (w/v) bavistin solution for 10 minutes to control fungal contaminants. After that, seedlings were transferred to three different potting media *viz*., i) brick chips: charcoal: sphagnum moss (1:1:2); ii) brick chips: charcoal: coconut husk (1:1:2) and iii) brick chips: charcoal: Leaf mould (1:1:1). The seedlings were covered with transparent plastic sheets for at least 2 weeks to maintained humidity and temperature and gradually removed to reduce humidity. Spraying of 1% bavistin twice a week was done to keep fungus away from the seedlings.

Data analysis

The experiments were done with 10 replications per treatment and repeated twice. The results were deliberated as mean ± standard deviation by application of Microsoft excels software.

Result and Discussion

Seed germination and protocorm formation

During the investigation, the effects of various basal medium, plant growth regulators and natural additives on organogenesis in all the species have been thoroughly studied. The mature seeds were inoculated in three different medium *viz*. MS, ½ strength of MS, IY and Mitra *et al*. as basal medium. In the present study MS media exhibited the best proliferation followed by Mitra *et al*. medium. The highest germination percentage of *Aerides ultiflora, Cleisocentron pallens* and *Rhynchostylis retusa* were observed in Mitra *et al*. medium 95.25%, 89.35% and 85% respectively. However, the other seven species exhibited better germination in MS medium. Previously, positive influence of basal MS medium on seed germination was also reported in *Esmeralda clarkei* (Paudal and Pant, 2012) [5] and *Cymbidium mastersii* (Mohanty *et al*. 2012) [6]. Highest germination percentage in Mitra *et al*. medium was also reported in *Cymbidium alloifolium* also (Nongdam & Chongtham, 2011) [7]. Successful germination was observed after 3-5 weeks of culture in different medium, evidence by the enlargement of the embryos and ultimately produces irregular shaped parenchymatous cell mass sphurels. The sphurels were transformed into round, oval, elongated, branched or spindle shaped protocorm after five to eight weeks of culture (Table-1). Lower amount of BAP was found most effective for transforming the spherules into fully developed protocorm like bodies (PLBs). Park *et al*. 2002 [8] reported BAP as a better phytohormone for PLB formation of *Phalaenopsis* spp.

Table 1: Germination medium and time taken for protocorm development

Name of Species	Media	Germination %	Spherule formation(in week)	Protocorm formation(in week)
Aerides multiflora	Mitra	95.25%	3-4	5-7
Cleisocentron pallens	Mitra	89.35%	3	5
Cymbidium aloifolium	MS	95.34%	3-4	5-7
Dendrobium aduncum	MS	90.43%	3-4	5-6
Dendrobium fimbriatum	MS	85.12%	3-5	6-8
Dendrobium lituiflorum	MS	95.48%	3-4	5-7
Dendrobium moschatum	MS	87.22%	3-5	6-8
Phalaenopsis mannii	MS	88.6	3	5
Phaius tankerville	MS	89.5%	3-4	5-7
Rhynchostylis retusa	Mitra	85%	3-4	5-7

Shoot formation and plantlet regeneration

On the basis of germination percentage, 7-week-old PLB's were transferred to Mitra *et al*. medium supplemented with different growth regulators like BAP (0.5, 1, 1.5, 2 mgL^{-1}), NAA (0.5mgL^{-1}) and IBA at various concentration either in individual or in combination to observed shoot development

for *A. multiflora, C. pallens* and *R. retusa*. Maximum number (3.9±0.88) of shoot with (5.56±0.58) length of *A. multiflora* was found in the medium supplemented with 1.5, mgL^{-1} BAP. The highest number and length of shoot (3.8±1.0 and 6.13±1.21cm. respectively) were recorded in 1.5 mg L^{-1} BAP and 0.5 mgL^{-1} NAA after 9 week of the culture. Maximum number (11.2±1.75, 4.1±0.74) and elongation rate of (4.75±0.58 cm, 5.13±0.85 cm) of shoot in 1.5 mgL^{-1} BAP and 0.5 mgL^{-1} IBA after 8 week of the culture for *C. pallens* and *R. retusa* respectively. In combination with 0.5mgL^{-1} IBA exhibited multiple shoot formation that favored root initiation also. Present study revealed that highest number (17.5 ±3.12) and length of shoot (6.01±0.66) was in the medium supplemented with 1.5, mgL^{-1} BAP. Seven week old PLB's of *C. aloifolium* was transferred to MS medium supplemented with BAP (1.5mgL^{-1}) showed highest number of shoot formation. Medium supplemented with 0.2% Activated Charcoal Powder (AC) play an active role in shoot formation and plantlet regeneration. 0.2% potato extract (PE), 0.2% yeast extract (YE) and 0.2% banana extract (BE) as natural additives also found excellent in *D. aduncum, D. fimbriatum* and *D. lituiflorum.* PLB's transferred to MS medium supplemented with TDZ (1.5 mgL^{-1}) was better phytohormons for shoot elongation of *D. moschatum*. The highest number and length of shoot 15.5 ±3.52 and 5.01±0.46cm were recorded in 1.5 mg L^{-1} TDZ after 9 week of the culture. Similar observations were also reported by Nayak *et al.* 1997 [9] in some *Dendrobium* species. *P. mannii* and *P. tankerville* BAP (1.5 mg L^{-1}) showed better response in shoot formation and elongation when the medium supplemented with 0.2% AC (12.5 ±3.62, 4.01±0.66 cm. and 15.5 ±3.62, 6.01±0.66 cm. respectively) (Table-2). Combined effect of NAA and BAP was more effective for multiple shoot formation of than the single effect in *A. multiflora* and *D. fimbriatum.* Ahmed (1996) [10] reported similar result for shoot multiplication in *Rhynchostylis retusa.* AC plays an important role in growth and development of some orchid species. It helps for absorbing the toxic substance released in the medium, aeration and light absorption. The positive response of AC powder was also reported in *phaleonopsis* (Hinnen *et al.*, 1989) [11]. Natural additives like BA, PA and YA were used for the regeneration of different *Dendrobium* species. BA was better in root formation of *D. lituiflorum* as well as YE showed better response in shooting and root multiplication. Earlier Vyas *et al.* (2009) [12] reported rapid proliferation of *D. lituiflorum* by the use of BE. Ac and PA were used in the regeneration and root formation of *D. aduncum*. Formerly, Roy *et al.* (2007) [13] and Shu *et al.* (2004) [14] reported in *D. chrysotoxum* and *D. tosaense.*

Table 2: Effect of plant growth regulator on shoot formation and plantlet regeneration after 9-12 week of culture

Name of Species	Medium	Growth regulators mgL^{-1}		Natural additives	Average number of shoot	Average length of shoot (cm.)
Aerides multiflora	Mitra	BAP	1.5	0.2% AC	3.9±0.88	5.56±0.58
		BAP+NAA	1.5+0.5		3.8±1.0	6.13±1.21
Cleisocentron pallens	Mitra	BAP+IBA	1.5+0.5	0.2% AC	11.2±1.75	4.75±0.58
Cymbidium aloifolium	MS	BAP	1.5	0.2% AC	17.5±3.12	6.01±0.66
		BAP+ IBA	1.5+0.5		12.5±1.50	6.78±0.46
Dendrobium aduncum	MS	BAP	1.5	0.2% PE	14.5±1.77	4.84±0.58
Dendrobium fimbriatum	MS	BAP	1.5	0.2% YE	7.9±0.98	5.76±0.48
		BAP+NAA	1.5+0.5		11.2±1.75	4.75±0.58
Dendrobium lituiflorum	MS	BAP	1.5	0.2% BE	12.5±1.77	5.78±0.46
		BAP+ IBA	1.5+0.5		15.2±1.75	4.75±0.58
Dendrobium moschatum	MS	TDZ	1.5	-	15.5 ±3.52	5.01±0.46
Phalaenopsis mannii	MS	BAP	1.5	0.2% AC	12.5 ±3.62	4.01±0.66
Phaius tankerville	MS	BAP	1.5	-	15.5 ±3.62	6.01±0.66
Rhynchostylis retusa	Mitra	BAP	1.5	0.2% AC	4.1±0.74	5.13±0.85
		BAP+ IBA	1.5+0.5		7.4 ± 1.07	3.43±0.85

Result based on average of 10 replicate per treatment denotes mean and ± standard deviation. AC: Activated Charcoal powder BE: Banana Extract YE: Yeast Extract PE: Potato Extract

In vitro root formation

For root initiation and development, the individual shoot bearing 4-5 cm long were selected. Majority of the species performed better root formation when the well-developed shoots were transferred to root inducing MS medium supplemented by IBA (1.5 mgL^{-1}) with 0.2% AC (Table-3). While, in *D. fimbriatum* the medium supplemented by NAA (1.5 mgL^{-1}) with 0.2% YE showed maximum number of root formation (7.1±1.24) with 6.75±0.87cm length. Highest root formation was recorded by the application of PE for *D. aduncum* and BE for *D. lituiflorum* as natural additives (Table-3).

Table 3: Effect of different plant growth regulator in multiple root development

Name of Species	Medium	Growth regulators (1.5 mgL^{-1})	Natural additives	Average number of root/shoot	Average length of root (cm.)
Aerides multiflora	Mitra	IBA	0.2% AC	4.6±0.97	6.5±0.45
Cleisocentron pallens	Mitra	IBA	0.2% AC	7.3±1.33	5.67±1.71
Cymbidium aloifolium	MS	IBA	0.2% AC	4.6±0.97	6.5±0.45
Dendrobium aduncum	MS	IBA	0.2% PE	7.9±1.33	4.6±1.71
Dendrobium fimbriatum	MS	NAA	0.2% YE	7.1±1.24	6.75±0.87
Dendrobium lituiflorum	MS	IBA	0.2% BE	5.9±0.95	6.5±0.45
Dendrobium moschatum	MS	IBA	0.2% AC	5.45±0.38	6.2±0.69
Phalaenopsis mannii	MS	IBA	0.2% AC	4.4±0.69	5.85±0.95
Phaius tankerville	MS	IBA	-	5.8±0.64	6.87±0.99
Rhynchostylis retusa	MS	IBA	0.2% AC	4.4±0.69	5.85±0.95

Result based on average of 10 replicate per treatment denotes mean and ± standard deviation. AC: Activated Charcoal powder BE: Banana Extract YE: Yeast Extract PE: Potato Extract

Acclimatization and Hardening

The plantlets of 4-6 cm. shoot length and well developed root were transferred to greenhouse condition after acclimatization with three potting medium at various concentration. The seedlings showed higher performance in the medium containing Brick chips: Charcoal: Sphagnum moss (1:1:2). However, Brick chips: Charcoal: Coconut husk (1:1:2) is best for *D. lituiflorum* and Brick chips: Charcoal: Sphagnum moss (1:1:2) for *R. retusa* (Table-4).

Table 4: Effect of best potting media on growth and development of plantlets

Name of Species	Composition of potting media	Survival rate	Average leaf number	Average leaf length (cm)	Average leaf width (cm)
Aerides multiflora	Brick chips : Charcoal: Sphagnum moss (1:1:2)	94%	5.4±1.14	3.34±0.44	1.65±0.49
Cleisocentron pallens	Brick chips : Charcoal: Sphagnum moss(1:1:2)	95	5.8±0.83	3.35±1.07	1.35±0.42
Cymbidium aloifolium	Brick chips : Charcoal: sphagnum moss (1:1:2)	95%	5.4±1.14	3.34±0.44	1.65±0.49
Dendrobium aduncum	Brick chips : Charcoal: Sphagnum moss (1:1:2)	91.9%	6.98±1.14	6.54±1.24	1.11±0.54
Dendrobium fimbriatum	Brick chips : Charcoal: Sphagnum moss (1:1:2)	89.34%	5.7±1.14	5.14±0.34	1.05±0.49
Dendrobium lituiflorum	Brick chips : Charcoal: Coconut husk (1:1:2)	90.34%	7.2±1.48	6.34±0.44	1.25±0.20
Dendrobium moschatum	Brick chips : Charcoal: Sphagnum moss (1:1:2)	90%	6.87±1.24	4.2±0.64	1.45±0.49
Phalaenopsis mannii	Brick chips Charcoal: Sphagnum moss (1:1:2)	90%	5.4±1.14	3.34±0.44	1.65±0.49
Phaius tankerville	Brick chips Charcoal: Sphagnum moss (1:1:2)	90%	5.4±1.14	3.34±0.44	1.65±0.49
Rhynchostylis retusa	Brick chips : Charcoal: Leaf mould (1:1:2)	90%	5.35±0.98	1.24±0.21	1.46±0.1

Result based on average of 10 replicate per treatment denotes mean and ± standard deviation.

Plate-I

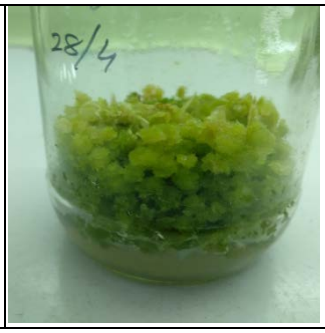
5				

Plate 2

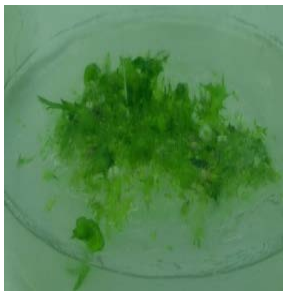
	A	B	C	D
6				
7				
8				
9				
10				

Plate 1 and 2: *In vitro* seed propagation of certain magnificent Orchids of Northeast India:

1. *Aerides multiflora*; 2. *Cleisocentron pallens*; 3. *Cymbidium aloifolium*; 4. *Dendrobium aduncum*; 5.*Dendrobium fimbriatum*; 6. *Dendrobium lituiflorum*; 7.*Dendrobium moschatum*; 8.*Phalaenopsis mannii*; 9. *Phaius tankerville* and *Rhynchostylis retusa.*
A: Protocorm formation; B: Well developed root & shoot; C: Best potting media D: Mass muliplication

Conclusion

The studied orchids are most beautiful and having immense commercial value. *In vitro* seed derived propagation technique is a simple approach for multiplication of orchids. It could be effectively applied for large scale propagation intended for future conservation, reintroduction in their natural ecological niche and mercantile aspects.

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