

## Effects of different factors on immature embryo culture, PLBs differentiation and rapid mass multiplication of *Coelogyne suaveolens* (Lindl.) Hook

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*In vitro* mass production of *C. suaveolens* (Lindl.) Hook, an endangered orchid with its snowy white flowers having horticultural potential was accomplished through immature seed culture, and subsequent plant regeneration. The developmental stage of the immature seeds and nutrient media significantly influenced the germination frequency. Seeds at 13 months after pollination cultured on 3% sucrose containing Murashige and Skoog (MS) medium with 9  $\mu$ M  $\alpha$ -naphthaleneacetic acid (NAA), and 15% coconut water exhibited 93% germination after 40 days of culture. Upon subculture, the germinated shoots on MS medium with 9  $\mu$ M BA, 6  $\mu$ M NAA, 3% casein hydrolysate and 0.1% activated charcoal (AC) yielded >12 shoots per shoot or bud. Addition of AC favoured the enlargement of pseudobulbs and better rooting. The plantlets transferred to community potting mix after *in vitro* hardening (8-10 wk) displayed 85% survival.

**Keywords:** Endangered epiphytic orchid, Immature seed culture, *In vitro* multiplication, Seed age

The orchids are propagated by vegetative means as well as through seeds. The rate of vegetative propagation is very slow and seed germination in nature is also very poor i.e., 0.2-0.3%<sup>1</sup>. *In vitro* germination of orchid seeds is an important part of the conservation and multiplication programme, as the 'dust seeds' are tiny and contain few food reserves. Knudson<sup>2</sup> demonstrated the possibility of by passing the fungal requirement of orchid seeds during *in vitro* germination and since then non-symbiotic seed germination has been accepted as an important tool for propagating orchids<sup>3</sup>. The non-symbiotic germination potential of fertilized ovules has been positively tested in several commercially viable and/or threatened orchid taxa<sup>4-8</sup>. Non-symbiotic seed germination of orchids are greatly influenced by several factors like seed age, different nutrient media with adjuvant and plant growth regulators<sup>2,4-9</sup>.

The genus *Coelogyne* is a medicinally important orchid and some species shown to possess some medicinally important compounds<sup>10</sup>. The species *C. suaveolens* (Lindl.) Hook, a sympodial epiphytic orchid and native of primary forests with attractive

snowy white flowers. From Nagaland the species was reported by Deb *et al*<sup>11</sup>. The distribution of this species restricted to some states of North-East India. In Nagaland only in few patches the presence of the species is recorded where the population in the natural habitat is very thin. Multiplication of this species in its natural habitat is very slow and the species is under threat in its natural habitat due to unregulated anthropogenic activities and destruction of forest cover. In this communication the effect of different factors like green pod age, culture media, and plant growth regulators etc have been studied on non-symbiotic seed germination and rapid mass multiplication.

### Materials and Methods

*Explant source and sterilization of the explants*—The cultures were initiated using immature seeds/embryos of different developmental stages at two wk interval from six months after pollination (MAP) to 18 MAP. The seeds were thoroughly scrubbed with liquid laboratory detergent (1:100 ratio, v/v) before washing with running tap water. The green pods were sterilized by dipping in 0.5% (w/v) aqueous solution of HgCl<sub>2</sub> for 5 min and subsequently rinsed repeatedly 4 or 5 times with sterilized distilled water. Finally, the green pods were flamed before scooping out the seeds in a laminar flow cabinet.

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**Culture media and Initiation of culture**—Three different basal media were tested for the present study viz. MS<sup>12</sup>, Mitra *et al.*<sup>13</sup> and Knudson 'C'<sup>14</sup> media. All the media were fortified with sucrose (0-4%) (w/v) as organic carbon source, coconut water (CW) (0-20%) (v/v), citric acid (100 mg l<sup>-1</sup>) as antioxidant. For seed germination all the media were further supplemented with different quantity of plant growth regulators (PGRs) like  $\alpha$ -naphthalene acidic acid (NAA) and N<sup>6</sup>-benzyl adenine (BA) (0-9  $\mu$ M) either singly or in combination. Difco-bacto agar (0.8%) was used as gelling agent and the pH of the media was adjusted to 5.6 using 0.1 N NaOH and 0.1 N HCl. About 15 ml of medium was dispensed in each test tube (size: 25×150 mm) and plugged before autoclaving at 1.05 kg cm<sup>-2</sup> pressure and at 121°C for 20 min.

The immature seeds at different MAP scooped out from the sterilized green pods and inoculated on different culture media. All the cultures were maintained at 25 $\pm$ 2°C under cool white fluorescent light at 40  $\mu$ M m<sup>-2</sup> s<sup>-1</sup> and 12/12 h light/dark photoperiod. The cultures were monitored regularly and the data were scored at two wk interval. The seeds were allowed to germinate and differentiate into protocorm like bodies (PLBs) on the same initiation medium. The germinated seeds/PLBs were maintained for another two passages on optimum culture conditions for further differentiation. Cultures were subcultured at four wk interval until mentioned otherwise.

**Culture differentiation, plantlets regeneration and mass multiplication**—The PLBs developed from the cultured immature seeds maintained for two passages on optimum germination medium for further differentiation. The germinated embryos/advance stage PLBs (with first set of leaflets) were transferred on MS medium containing sucrose (0-3%), casein-hydrolysate (CH) (0-0.15%) (v/w), different levels of PGRs viz. NAA, IAA, BA and KN (0-9  $\mu$ M) either singly or in combination for mass multiplication and regeneration of plantlets. Activated charcoal (AC) (0-0.3%) (w/v) was also incorporated in the regeneration medium to study the effect on morphogenetic response and mass multiplication. The shoot buds and regenerated plants were maintained for 2-3 passages for mass multiplication.

**Hardening of plants and transferring to potting mix**—The plantlets of 3 or 4 cm height with 3-4 leaves and 3 or 4 roots with distinct pseudobulb from the regeneration medium were used for the hardening.

The traces of agar in the roots were washed off with double distilled water before hardening. The plantlets were maintained in culture vials containing 1/10<sup>th</sup> MS basal liquid medium with sucrose (2%). Sterilized small pieces of sterile charcoal, decay sawdust, brick pieces and chopped litter (1:1:1:1 ratio) were also incorporated as substratum in the culture vial and maintained for 8-9 wk before transferring to potting mix. The transplants were maintained the poly house for 2 months before transferring to the wild.

The experimental design was completely randomized. For every treatment 15 culture vials were maintained and the experiments were repeated at least thrice.

## Results and Discussion

**Seed germination and PLBs formation**—During the preliminary *in vitro* studies, the seeds at an age of 13 MAP responded well to MS medium containing 9  $\mu$ M BA and 3  $\mu$ M NAA. Thus further study using seeds at different age was performed to find out the optimal age of the seeds by culturing on these media and was followed by refinement of optimal media using seeds at optimal age. After 25-30 days of inoculation the first sign of germination was recorded as greening and nodular swelling of the cultured seeds (Fig. 1 a). The Swelled seeds then converted into PLBs after 40 days of inoculation on germination medium (Fig. 1 b). For successful culture initiation the age of the green pod was found to be critical factor. The green pod age/seed age up to 8 MAP did not support germination, while seeds >14 MAP shows low percentage and deformed germination. The optimum germination was recorded with seed age of 13 MAP where within 23 days of culture the cultured seeds started greening about 93% response was recorded after 40 days of culture (Table 1). The importance of time interval between pollination and fertilization has been stressed and varies with different species. In *Cleisostoma racemiferum*<sup>8</sup> green pod age of 16 week after pollination (WAP) was found to be suitable for healthy culture initiation, while 16-18 WAP in *Arachnis labrosa*<sup>7</sup>, 8-9 WAP in *Malaxis khasiana*<sup>6</sup>, 120 days *Cymbidium macrorhizon*<sup>15</sup> and in *C. Iridiodes*<sup>9</sup>.

Considering the previous reports on the effectiveness of various basal media for orchid seeds germination of *Cleisostoma racemiferum*<sup>8</sup>, *Malaxis khasiana*<sup>6</sup>, *Arachnis labrosa*<sup>7</sup> and *Cymbidium macrorhizon*<sup>15</sup>, the 3 basal media with optimal levels

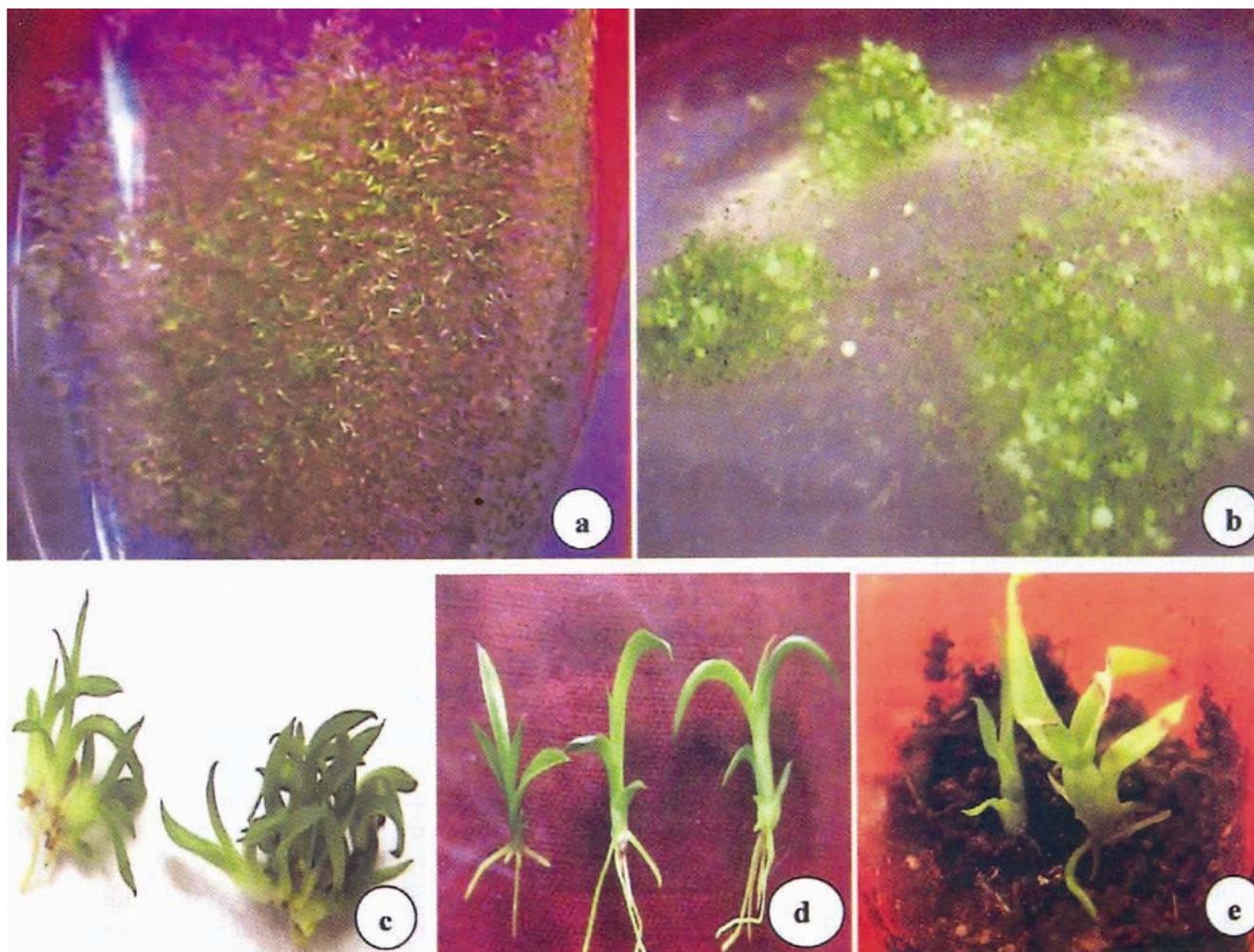


Fig. 1—Asymbiotic seed germination and plant regeneration of *C. suaveolens*. [a. Germination of immature seeds (13 MAP) on MS medium with 3% sucrose, 15% CW, 3  $\mu$ M NAA and 9  $\mu$ M BA ; b. PLBs formed from the cultured seeds (13 MAP); c. Multiple shoots development on MS medium containing 6  $\mu$ M NAA + 9  $\mu$ M BA, 3% sucrose, 0.1% AC and 0.1% CH; d. Rooted plants ready for hardening, and e. Plantlets in hardening condition].

Table 1—Effect of seed age on immature seed germination<sup>#</sup> of *C. suaveolens*

Seed age (MAP)	Days taken for			Germination (%) ( $\pm$ SE) *	Types of response
	Greening	Germination	1 <sup>st</sup> leaflet		
9	60	82	97	50 ( $\pm$ 0.5)	Nodular swelling followed by few PLBs formation
10	41	61	73	61 ( $\pm$ 0.5)	As above
11	34	55	70	72 ( $\pm$ 0.75)	As above
12	25	45	60	80 ( $\pm$ 1.0)	Nodular swelling followed by healthy PLBs formation
13	23	40	56	93 ( $\pm$ 1.0)	Green PLBs and healthy plantlets formation
14	27	47	62	76 ( $\pm$ 1.0)	Nodular swelling followed by healthy plantlets formation
15	32	51	67	65 ( $\pm$ 0.5)	As above
16	45	66	81	60 ( $\pm$ 1.0)	Nodular swelling followed by few plantlets formation
17	52	73	88	50 ( $\pm$ 1.25)	As above
18	66	87	102	42 ( $\pm$ 1.0)	Deformed and delayed germination and fewer PLBs formed

4 to 8 MAP seed age not presented as there was no response

<sup>#</sup> On MS medium containing 3% sucrose 15% CW, 3  $\mu$ M NAA and 9  $\mu$ M BA

\* Standard error.

Data represent the mean of three replicates

of BA (9  $\mu\text{M}$ ) and NAA (3  $\mu\text{M}$ ) were attempted for the present study (Table 2). The culture of immature seeds at an optimal age (13 MAP) showed significant differences in response as to the 3 basal media (Table 2). Of the 3 media, MS medium supported the best germination (93%) (after 40 days of culture) and followed by Mitra *et al.* (68%) (after 48 days of culture) and Knudson 'C' (45%) (after 52 days of culture). Further MS medium favoured formation of healthy PLBs, and subsequently healthy plantlets with distinct pseudobulbs. The cultures upon Mitra *et al.* medium the PLBs were unhealthy and the plantlets showed etiolation and fewer shoot. On Knudson 'C' medium the PLBs formation was arrested right after nodular swelling of the seeds and remained pale yellow color and degenerated subsequently.

On media devoid of sucrose no germination was registered. Murashige and Skoog medium with BA and NAA + 3% sucrose + 15% CW exhibited optimum response. It was observed that the germination percentage declined considerable at higher or at lower concentration. At lower concentration of sucrose (2%) produced unhealthy PLBs and germination duration prolonged while, medium with sucrose (4%) germination percentage decreases and results with stunted growth. Though incorporation of CW in the germination medium was not prerequisite, incorporation of CW (15%) in the medium enhanced the early germination and differentiation of PLBs into healthy plantlets. While at higher or at lower concentration of CW decreased in germination rate.

Amongst the different quantity of PGRs tested in the present study a combined treatment of NAA and BA was found to be most suitable over other treatments. When used singly, they either delayed or lowered the germination percentage. Optimum

germination was observed on medium containing 3  $\mu\text{M}$  NAA + 9  $\mu\text{M}$  BA in combination followed by 3  $\mu\text{M}$  NAA + 6  $\mu\text{M}$  BA in combination (Table 3). The synergistic effect of NAA and BA in non-symbiotic seed germination as in the present study has been reported in *C. racemiferum*<sup>8</sup> where NAA + BA in combination superior over other treatments. However, combined treatment of NAA (0.1-0.5  $\text{mg l}^{-1}$ ) + KN (1-2  $\text{mg l}^{-1}$ ) favoured optimum germination in *Vanda coerulea*<sup>5</sup>.

*PLBs differentiation, plant regeneration and mass multiplication*—Maintenance of the germinated seeds on the optimal germination medium for two more passages favoured PLBs formation, differentiation and subsequent shoot multiplication. The advance stages of PLBs were maintained on regeneration medium. Within 8-9 wk of culture on MS medium with 9  $\mu\text{M}$  and 6  $\mu\text{M}$  NAA facilitated shoot formation and multiplication at the rate of 12 shoots/buds per explants (Fig. 1 c). Both auxin and cytokinin when used singly did not support healthy culture or differentiation. A singly treatment of IAA favoured stunted growth and KN resulted etiolation of the plantlets. Incorporation of AC (0.1%) and CH (0.1%) in the regeneration medium triggered early differentiation of shoots and roots with distinct pseudobulbs development (Table 4). Presence of AC (0.1%) in the medium helps in early initiation of roots and activates shoot buds formation accompanied by healthy growth of plantlets. Within 8-9 wk of culture on regeneration medium the plantlets attained a height of ~ 3.5 cm with 3 or 4 roots (Fig. 1 d). The positive effect of cytokinin and auxin in PLBs formation and plantlet regeneration as in the present study has been reported in *Malaxis khasiana*<sup>6</sup> and *M. accuminata*<sup>16</sup>. The promontory effect of AC on plant regeneration, mass multiplication and rhizome enlargement were

Table 2—Effect of media composition in seed germination of *C. suaveolens*

Basal media <sup>#</sup>	Germination duration (days)	Germination rate (%) ( $\pm$ SE)*	Type of response
Mitra <i>et al.</i> medium	48	68 ( $\pm$ 1.5)	Nodular swelling followed by PLBs formation but conversion of PLBs into plantlets delayed.
MS	40	93 ( $\pm$ 1.0)	All the PLBs were green and converted into healthy plantlets.
Knudson 'C'	52	45 ( $\pm$ 0.5)	Nodular swellings but failed to converting to healthy PLBs, culture remained pale yellow and subsequently degenerated after browning.

<sup>#</sup> Media containing 3  $\mu\text{M}$  NAA + 9  $\mu\text{M}$  BA, 3% sucrose and 15% CW  
\* Standard error.  
Data represent the mean of three replicates

Table 3—Effect of BA and NAA on germination of seeds (13 MAP) of *C. suaveolens*<sup>#</sup>

PGRs conc. ( $\mu\text{M}$ )		Days taken to germinate	Germination rate (%) ( $\pm\text{SE}$ )*	Type of response
NAA	BA			
0	0	—	—	No response
3	0	68	47 ( $\pm 1.5$ )	Few PLBs
6	0	63	48 ( $\pm 1.0$ )	As above
9	0	60	45 ( $\pm 1.5$ )	As above
0	3	57	50 ( $\pm 1.0$ )	Few green PLBs
0	6	55	60 ( $\pm 1.5$ )	As above
0	9	55	65 ( $\pm 2.0$ )	Healthy green PLBs
3	3	53	70 ( $\pm 1.0$ )	Few PLBs
3	6	46	75 ( $\pm 2.0$ )	Healthy green PLBs formed
3	9	40	93 ( $\pm 1.5$ )	Green and distinct PLBs followed by healthy plantlets
6	3	48	46 ( $\pm 1.5$ )	Few green PLBs
6	6	53	50 ( $\pm 1.5$ )	As above
6	9	53	70 ( $\pm 2.5$ )	As above
9	3	61	45 ( $\pm 1.0$ )	Few PLBs
9	6	66	50 ( $\pm 1.0$ )	As above
9	9	72	55 ( $\pm 1.5$ )	As above

<sup>#</sup> On MS medium containing sucrose (3%), CW (15%)

\* Standard error

Data represent the mean of three replicates

Table 4—Effect of AC and CH on plant regeneration and mass multiplication of *C. suaveolens*<sup>#</sup>

AC conc. (%)	CH conc. (%)	Type of response*		
		Shoot height (cm) ( $\pm\text{SE}$ )**	Nos. of roots ( $\pm\text{SE}$ )**	Nos. of shoot buds ( $\pm\text{SE}$ )**
0	0	0.8 ( $\pm 0.33$ )	2 ( $\pm 0.33$ )	2 ( $\pm 0.33$ )
0.05	—	1.0 ( $\pm 0.25$ )	2 ( $\pm 0.33$ )	4 ( $\pm 0.5$ )
0.1	—	2.0 ( $\pm 0.33$ )	3 ( $\pm 0.33$ )	6 ( $\pm 0.5$ )
0.15	—	1.6 ( $\pm 0.33$ )	3 ( $\pm 0.33$ )	4 ( $\pm 0.5$ )
0.2	—	1.0 ( $\pm 0.25$ )	3 ( $\pm 0.33$ )	3 ( $\pm 0.33$ )
0.25	—	1.0 ( $\pm 0.33$ )	2 ( $\pm 0.25$ )	2 ( $\pm 0.33$ )
0.3	—	0.8 ( $\pm 0.33$ )	1 ( $\pm 0.25$ )	2 ( $\pm 0.5$ )
—	0.05	1.6 ( $\pm 0.25$ )	3 ( $\pm 0.25$ )	7 ( $\pm 0.33$ )
—	0.1	2.0 ( $\pm 0.33$ )	3 ( $\pm 0.33$ )	8 ( $\pm 0.33$ )
—	0.15	1.5 ( $\pm 0.2$ )	2 ( $\pm 0.25$ )	4 ( $\pm 0.25$ )
0.1	0.05	1.7 ( $\pm 0.2$ )	3 ( $\pm 0.3$ )	10 ( $\pm 0.04$ )
0.1	0.1	3.5 ( $\pm 0.33$ )	4 ( $\pm 0.25$ )	12 ( $\pm 0.5$ )
0.1	0.15	2.0 ( $\pm 0.2$ )	3 ( $\pm 0.3$ )	8 ( $\pm 0.3$ )

Note: Only the responding treatments are computed in the presentation.

<sup>#</sup> On MS medium containing NAA+BA (6+9  $\mu\text{M}$  respectively), sucrose (3%)

\* Data represent the mean of three replicates and scored after 8 wk of culture on the medium

\*\* Standard error

also reported in *M. khasiana*<sup>6</sup>, but in *M. accuminata*<sup>16</sup> incorporation of AC was found inhibitory.

**Hardening of plants and transferring to community potting mix**—After maintaining 2-3 passages on the regeneration medium the plantlets about 3-4 cm with 3-4 roots with distinct pseudobulb were transferred to hardening medium as described in material and method (Fig. 1 e). Hardened plants were transferred to community potting mix. The potted plants were maintained in the poly-house for ~8-9 wk before transferring to the wild. About 80-85% of survival is registered after two months of transfer.

The protocol describes for rapid mass multiplication of this elegant orchid whose population is under check in its natural habitat. The protocol could also be useful for *in vitro* short-to medium-term conservation of the species.

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#### References

- 1 Vij S P, Orchids and tissue culture: Current status, in *Role of plant tissue culture in biodiversity conservation and*

- economic development*, edited by S K Nandi, L M S Palni & A Kumar (Gyanodaya Prakashan, Nainital, India) 2002, 491.
- 2 Knudson L, Non-symbiotic germination of orchid seeds, *Bot Gaz*, 73 (1922) 1.
  - 3 Arditti J, Clements M A, Fast G, Hadley G, Nishimura G & Ernst R, Orchid seed germination and seedling culture – A manual, in *Orchid Biology – Reviews and perspectives*, Vol. II edited by Arditti J (Cornell University Press, Ithaca, New York) 1982, 243.
  - 4 Sharma S K & Tandon P, Asymbiotic germination and seedling growth of *Cymbidium elegans* Lindl. and *Coelogyne punctulata* Lindl. as influenced by different carbon sources, *J Orchid Soc India*, 12 (1990) 83.
  - 5 Devi C G, Damayanti M & Sharma G J, Aseptic embryo culture of *Vanda coerulea* Grief, *J Orchid Soc India*, 12 (1998) 83.
  - 6 Deb C R & Temjensangba, *In vitro* propagation of threatened terrestrial orchid, *Malaxis khasiana* Soland ex. Swart, *Indian J Exp Biol*, 44 (2006) 762.
  - 7 Temjensangba & Deb C R, Regeneration and mass multiplication of *Arachnis labrosa* (Lind. Ex Paxt.) Reichb: A rare and threatened orchid, *Curr Sci*, 88 (2005) 1966.
  - 8 Temjensangba & Deb C R, Effect of different factors on non-symbiotic seed germination, PLBs formation and plantlet morphology of *Cleisostoma racemiferum*, *Indian J Biotech*, 5 (2006) 223.
  - 9 Jamir C, Devi J & Deka P C, *In vitro* propagation of *Cymbidium iridioides* and *C. lowianum*, *J. Orchid Soc India*, 16 (2002) 83.
  - 10 Majumder P L, Chakraborty S & Sen S, Stilbenoids and flavonoids from the orchids *Coelogyne uniflora*, *Ione paleacea* and *Acrochoene punctata*, *J Indian Chem Soc.*, 79(2) (2002) 169.
  - 11 Deb C R, Jamir, N S & Temjensangba, Orchid diversity of Nagaland – A revised status, *J Orchid Soc India*, 17 (2003) 5.
  - 12 Murashige T & Skoog K, A revised medium for rapid growth and bioassays with tobacco tissue culture, *Physiol Plantanum*, 15 (1962) 473.
  - 13 Mitra G C, Prasad R N & Roy Chowdhury A R, Inorganic salts and differentiation of protocorm in seed callus of an orchid and correlation changes in its free amino acid content, *Indian J Exp Biol*, 14 (1976) 350.
  - 14 Knudson L, A new nutrient solution for germination of orchid seeds, *American Orchid Soc Bull*, 15 (1946) 214.
  - 15 Vij S P & Pathak P, Asymbiotic germination of the saprophytic orchid, *Cymbidium macrorhizon*: A study *in vitro*, *J Orchid Soc India*, 2 (1988) 25.
  - 16 Vij S P & Kaur S, Micropropagation of the therapeutically important orchids: *Malaxis acuminata* D. Don, *J Orchid Soc India*, 12 (1998) 89.