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In vitro REGENERATION POTENTIAL OF FOLIAR EXPLANTS OF *Malaxis acuminata* D. Don.: A THERAPEUTICALLY IMPORTANT TERRESTRIAL ORCHID

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ABSTRACT

***Malaxis acuminata* is a terrestrial orchid whose rhizome is medicinally important. The dried pseudobulbs are used in preparation of 'Ayurvedic drug' '*Chyawanparash*', an energetic herbal tonic and to cure tuberculosis. Due to various anthropogenic activities and excessive extraction for medicinal purposes, the species is under constant threat in its natural habitats. A successful attempt was made to propagate this species using foliar explants from axenic source as alternative explants. The meristemetic loci invoked from the cultured leaf explants within 4-5 weeks of culture on MS medium fortified with sucrose (3%, w/v) and NAA (3 µM) + BA (3 µM). The foliar explants were cultured in different orientations, and optimum response was recorded with the explants cultured horizontally on culture medium in comparison to up side up and reverse orientations. Under this condition, as many as 26 shoot buds developed. The resultant shoot buds converted into plantlets with well developed roots on MS medium supplemented with sucrose (3%) and NAA + BA (3 µM each) with average plant height, number of leaves and number of shoots 2.4 cm, 4.5, 18.0, respectively. The hardened regenerates when transferred to community potting mix [prepared by mixing charcoal pieces, chopped forest litters, coconut husk, sand and black soil at 1:1 ratio] showed about 75% survival of transplants after two months.**

Key words: Foliar explants, *in vitro* propagation, leaf orientation, *Malaxis acuminata*, orchid.

INTRODUCTION

Clonal propagation of high value forest plants through organogenesis has the potential for rapid capture of the benefits of breeding programme thereby improve the quality and uniformity of stock plants (Lakshmanan *et al.*, 2006; Gopi and Ponmurugan, 2006; Arumugam *et al.*, 2009). With the refinement and advancement of plant tissue culture technology in horticultural crops, the attention is now focused on the use of resident meristems as a source of explants for *in vitro* propagation. Use of resident meristems like leaf, roots, etc. is advantageous because of their easy availability and ease in disinfection. Further, their excision does not endanger the mother plant (Temjensangba and Deb, 2005a; Deb and Temjensangba, 2005, 2006a; Deb and Arenmongla, 2011). As regeneration occurs in dermal cells, the cytologically more stable cells, the regenerates are genetically uniform (Vij, 2002). Regenerative competence of foliar explants has successfully been tested in some plant species (Seeni and Latha, 1992; Deb and Temjensangba, 2007a; Deb and Arenmongla, 2011) but in most of these cases the success is restricted to epiphytic orchids.

Orchids, besides their horticultural values, are therapeutically important sources of curare compounds to several ailments (Deb *et al.*, 2009). The genus *Malaxis*, a therapeutically important orchid, comprises of ~300 species of which 19 are reported from India (Deb and Temjensangba, 2006b). *Malaxis acuminata* D. Don is a therapeutically important species and is component of

'*Ayurvedic*' drug preparation. The dried pseudobulbs are important ingredient of '*Ashtavarga drug*' used in the preparation of '*Chyawanparash*', an energetic herbal tonic and to cure tuberculosis (Deb and Temjensangba, 2006b; Deb and Imchen, 2008; Deb *et al*., 2009). Due to the over-extraction of *M. acuminata* for medicinal purpose, the species growing under natural habitats is under constant threat. Since the natural population of this species is downsized on each passing day, it is essential to develop alternative propagation techniques for its rapid multiplication. The present study was aimed to investigate the influence of factors like PGR and explants orientation on culture medium and on the *in vitro* morphogenetic potential of foliar explants of *Malaxis acuminata*.

MATERIALS AND METHODS

Selection of explants

The stock cultures were raised by culturing immature embryos of 7-8 wk old on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 4 µM α-naphthalene acetic acid (NAA) where the germinated seeds formed protocorm-like bodies (PLBs) after 7-8 wk of culture (Arenmongla and Deb, 2012). The nodal explants were collected from these stock cultures, maintained in plant tissue culture laboratory of Nagaland University. The PLBs were converted into plantlets on MS medium enriched with 3% sucrose and 3 µM each of NAA and BA (in combination). Leaves were collected from 5-6 wk old plantlets from *in vitro*/axenic culture**.** After harvesting the leaves from *in vitro*-raised shoots, the leaves were soaked in sterilized distilled water till culture on initiation medium.

Tissue culture medium and initiation of culture

For the initiation of cultures, MS medium containing 0.8% agar (w/v) as gelling agent was fortified with 3% sucrose [as organic carbon source] and different concentrations of plant growth regulators (PGRs) viz., NAA and benzyl adenine (BA) (0 to 9 µM). The pH of medium was adjusted to 5.6 using 0.1N NaOH and 0.1N HCl. The medium (12 ml) was dispensed in each borosilicate test tube (25x150 mm), plugged with nonabsorbent cotton and autoclaved at 121^{o}C for 20 min. at a pressure of 1.05 kg cm^{-2}. After sterilization, the test tubes were slanted at ~45^{o} angle before setting the gel. The soaked leaves were cultured on initiation medium and for each treatment 20 leaves were used. The foliar explants were cultured in three different orientations viz., up side up (petiole part toward down), upside down (petiole side up i.e., reverse orientation) and horizontal.

The experiment was conducted in a completely randomized design and each experiment replicated 5 times. The cultures were maintained at 25±2^{o}C under cool white fluorescent light at 40 µmol m^{-2} s^{-1} and 12/12 light/dark photo period. All the cultures were sub-cultured at 4-5 wk interval unless mentioned otherwise. *In vitro* response was evaluated on the basis of percent explants responded and the number of propagules formed in culture after specific time. The data was expressed as the mean of replicates ± standard error.

The PLBs/shoot buds formed from the foliar explants were maintained for another two passages on optimum initiation condition for further differentiation [i.e. medium supplemented with optimum PGRs concentration(s) achieved from initiation medium].

Multiplication and plant regeneration

The young tiny shoot buds from the initiation medium were transferred on MS medium for regeneration and mass multiplication purpose. The medium was supplemented with 3% sucrose and PGRs *viz.*, NAA and BA (0 to 9 µM), used either individually or in combination. All the cultures were maintained at full laboratory illumination as mentioned earlier. The regenerated shoot buds/plantlets were separated from the clumps of produced shoot buds and transferred on fresh regeneration medium for culture proliferation and growth. The plantlets were maintained for 2-3 passages for further growth and differentiation on regeneration medium before transferring them for hardening.

Hardening & transplantation of the regenerates

The well rooted plantlets from regeneration medium were taken out carefully inside the laminar flow cabinet and cultured on ½ strength MS medium with 2% sucrose but devoid of any PGR under normal laboratory conditions for 6-7 wk prior transfer to potting mix. Then well hardened plantlets were taken out from the culture vials, the traces of agar removed carefully using a fine brush and transferred to plastic pots containing potting mix. The potting mix comprised of charcoal pieces, chopped forest litter, coconut husk, sand and black soil (in 1:2:1:1:2 ratio) with a moss topping. The pots were covered with holed transparent polybags and watered at weekly interval for one month. The plants were fed with $1/10^{th}$ MS salt solution at weekly interval for 3-4 wks and maintained in poly-shade with ca.70% of shading sunlight, before transferring them to the wild.

RESULTS AND DISCUSSION

Initiation of culture

The cultures were initiated from the foliar explants of ~5-6 wk old from *in vitro* source. The responsive leaves were identified by the formation of hairy structures at the petiole base/basal part of the foliar explants (Fig. 1a) followed by PLBs and shoot buds formation. The foliar explants were cultured in different orientations with the objective to study the effect of explants orientation on morphogenetic response. Of the different orientations, the explants placed horizontally on culture medium exhibited better morphogenetic response in comparison to up side up and reverse orientations (Table 1). After 4-5 wk of culture, meristematic activity was invoked at the basal ends of explants. Mostly meristematic activity was restricted towards the lower basal parts. Wimber (1965) successfully developed PLBs from the leaves of *Cymbidiums*, which opened up an effective alternative to apical shoot meristem culture. Since then the regenerative competence of foliar explants were positively tested for more than 60 orchid species (Temjensangba and Deb, 2005a; Deb and Sungkumlong, 2010). However, the success is restricted mostly with epiphytic orchids and only few species from terrestrial orchids suggesting thereby that the ground orchids are less amenable to *in vitro* regeneration (Deb and Sungkumlong, 2010). In present study, the initiation of morphogenetic response was restricted to the basal parts of foliar explants. The morphogenetic potential of leaf base has been reported in *Coelogyne*, *Dendrobium*, *Oncidium* and *Phalaenopsis* (Abdul Karim and Hairani, 1990), *Acampe praemorsa* (Nayak *et al*., 1997), *Arachnis labrosa* (Deb and Temjensangba, 2007a), *Cleisostoma racemiferum* (Temjensangba and Deb, 2005a), *Coelogyne suaveolens* and *Taenia latifolia* (Deb and Sungkumlong, 2010), *Vanda coerulea* (Vij and Aggarwal, 2003). Mathews and Rao (Mathews and Rao, 1985) considered the leaf base to be a decisive factor for culture initiation from foliar explants. Sinha and Hegde (1999) reported the development of meristematic activity along the entire leaf in Renades Arunoday Hybrid. Earlier, Nayak *et al.* (1997) reported remarkable influence of explants orientation of shoot development in *Acampe praemorsa*.

Table 1: Effect of leaf orientation on morphogenetic response of *Malaxis acuminat

Leaf orientation	No. of shoots formed (±SE)	% response (±SE)	Time for response (days)	Type of response
Upside up	18 (1.0)[b]	75 (2.0)[a]	38	Hairy structures, shoots small, developed from petiole
Upside down	12 (2.0)[c]	50 (2.5)[c]	46	Leaf curled, shoots small, unhealthy, degenerated gradually
Horizontal	26 (2.0)[a]	65 (2.0)[b]	28	Shoots developed from petiole, shoots healthy, hairy structures, shoots formed in clusters, healthy

The figures in the same column superscripted by the same alphabets are statistically identical to the threshold of 5% (Newman-Keuls, ±standard error).; *On MS medium with sucrose (3%) and NAA + BA (3 + 6 µM in combination); The data represents the mean of 5 replicates.

Fig. 1: **Different stages of morphogenetic response in foliar explants of *Malaxis acuminata*. a. Cultured leaf showing swelling at the basal part, b. Multiple shoot buds developed from the basal part of the cultured leaf, c. Multiple shoots/plants developed on regeneration medium, d. Acclimatized regenerates transferred in potting mix.**

The incorporation of PGRs to the basal medium was obligatory for the initiation of culture. The explants failed to respond when cultured on PGRs free medium. The role of growth hormones in stimulating meristematic activity and promoting proliferation in leaf explants is well documented in orchids (Temjensangba and Deb, 2005a; Deb and Temjensangba, 2006a, 2007b; Li and Xu, 2009). Murashige (1974) opined that *in vitro* plant regeneration occurs frequently through adventitious shoot formation and rarely through somatic embryogenesis. In general, amongst different quality and quantity PGRs tested for culture initiation, the single treatment of NAA and BA did not support healthy morphogenetic response. Single treatment with NAA did not support healthy organogenesis and in most cases the explants exhibited callusing while, lone treatment with BA supported moderate

Table 2: Effects of PGRs on morphogenetic response of foliar explants (5 wk old) of *M. acuminata* from *in vitro* source

PGR conc. (μM)		Time for initial response (days)	% response (±SE)	No. of shoots formed	Type of response*
NAA	BA				
0	0	-	-	-	No response
0	3	35	63 (1.0)[a]	25	Leaf curled followed by hairy structure formation; PLBs and shoots formed
0	6	47	30 (1.5)[e]	3	Curling of leaf; shoots developed from vein
0	9	52	45 (2.5)[d]	6	Leaf curled; PLBs and shoots developed from petiole; white in color; degenerated gradually
3	0	46	55 (2.5)[c]	15	Hairy structure; few healthy plantlets developed; shoots small
3	3	31	60 (1.7)[b]	21	Swelling at petiole; healthy shoots developed from petiole; roots developed
3	6	28	65 (1.5)[a]	26	Fewer PLBs formed; shoots healthy; hairy structures; shoots formed in clusters; healthy plantlets
3	9	34	60 (2.0)[b]	14	Shoots small; round structures; degenerated gradually
6	3	42	55 (2.0)[c]	8	Hairy structures; shoots small; developed from petiole
6	6	55	25 (1.0)[f]	0	Slight swelling; but gradually degenerated
6	9	55	45 (3.0)[d]	10	Shoots small
9	6	60	30 (2.0)[e]	3	Leaf curled; shoots small; unhealthy

The figures in the same column followed by the same alphabets are statistically identical to the threshold of 5% (Newman-Keuls, ± standard error); *On MS medium with sucrose (3%);
The data represents the mean of 5 replicates. The data collected after 50 days of culture.

organogenesis. The optimum morphogenetic response was recorded on medium enriched with 3 μM NAA and 6 μM BA in combination (Table 2). About 65% cultured explants responded positively after 28 days of culture on MS medium containing sucrose (3%) and 3 μM NAA + 6 μM BA where 26 meristemoids/shoot buds invoked after maintaining for two passages on medium fortified with optimum PGRs which subsequently converted into PLBs and shoot buds (Fig. 1b). However, at higher concentrations morphogenetic response declined significantly and in many cases explants degenerated. The advanced stage PLBs/shoot buds/tiny plantlets from initiation medium were maintained for another 2 passages for further differentiation and culture proliferation. The PLBs and shoot buds formed from the cultured foliar explants were transferred on regeneration medium for regeneration of plantlets and culture proliferation.

Multiplication and plant regeneration

For plantlets regeneration, incorporation of PGR was obligatory. Under the optimum regeneration condition as many as 18 shoots/shoot buds developed explants^{-1} subculture^{-1} (Table 3; Fig. 1c). The advanced stage PLBs/ plantlets started converting into young rooted plantlets and repetitive PLBs within 3-4 wk on regeneration medium. Amongst the different concentrations of NAA and BA tested, a combined treatment of NAA and BA (3 μM each) supported healthy regeneration and multiple shoot formation. Cultures with individual treatments of BA were better as compared to NAA. Cultures maintained on NAA alone supported more root formation but suppressed multiple shoot formation and leaf formation whereas the cultures on BA enriched media, although had moderately high number of shoots, did not support healthy root formation. When both NAA and BA were incorporated in combination, it supported healthy shoot formation with multiple roots. Chen *et al.* (2004), Temjensangba and Deb (2005b) and Pongener and Deb (2011a) have argued that the change in culture

Table 3: Effect of various levels of PGRs on plantlet regeneration and mass multiplication of *M. acuminata*

PGR conc. (µM)		Plantlet height (cm)*	No. of leaves	No. of shoots	Type of response**
NAA	BA				
0	3	2.1(±0.2)^{b}	3.0(±0.1)^{b}	3.2(±0.3)^{d}	Plantlet small, etiolated, leaves small, hairy structures
0	6	1.5(±0.2)^{c}	2.1(±0.3)^{c}	4.2(±0.2)^{d}	Plantlet thin, unhealthy
0	9	1.6(±0.3)^{c}	3.0(±0.2)^{b}	8.3(±0.4)^{c}	Stunted growth
3	0	3.4(±0.2)^{a}	3.1(±0.1)^{b}	2.0(±0.2)^{e}	Plantlet curled etiolated, thin, hairy structures.
6	0	3.2(±0.2)^{a}	3.0(±0.1)^{b}	3.1(±0.2)^{d}	Plantlets etiolated and few plantlet degenerated
9	0	1.1(±0.2)^{c}	2.0(±0.2)^{c}	2.1(±0.1)	Plantlet small, few shoots, slight pigmentation, few degenerated
3	3	2.4(±0.3)^{b}	4.5(±0.2)^{a}	18.2(±0.4)^{a}	Healthy plantlets with many shoot buds, violet pigmentations formed, pseudo-bulb swollen
3	6	1.9(±0.2)^{b}	3.2(±0.1)^{b}	6.1(±0.2)^{c}	Plantlet healthy, shoots formed in clusters, hairy structures, slight pigmentations.
3	9	1.8(±0.1)^{b}	2.1(±0.1)^{c}	3.0(±0.2)^{d}	Stunted growth, slight pigmentations formed at the base
6	3	2.9(±0.2)^{a}	4.2(±0.2)^{a}	9.0(±0.4)^{b}	Healthy plantlet, shoot buds elongated/ etiolated and formed in clusters, pigmentations formed, leaves light green
6	6	1.6(±0.2)^{c}	3.0(±0.2)^{b}	10.2(±0.5)^{b}	Plantlets etiolated, shoot buds formed in clusters, vitrified, degenerated subsequently, slight pigmentation.
6	9	1.9(±0.2)^{b}	3.1(±0.2)^{b}	8.4(±0.3)^{c}	Shoots formed in clusters, slight pigmentation, callused at the base
9	3	1.7(±0.2)^{c}	3.0(±0.3)^{b}	10.4(±0.4)^{b}	Healthy plantlets shoots formed in clusters, slight pigmentation, pseudobulb swollen
9	6	2.1(±0.1)^{b}	3.1(±0.1)^{b}	3.1(±0.2)^{d}	Shoot buds very small
9	9	1.8(±0.2)^{b}	2.0(±0.2)^{c}	8.4(±0.2)^{c}	Shoot buds small and formed in clusters, plantlet unhealthy, slight pigmentation

The figures in the same column superscripted by the same alphabets are statistically identical to the threshold of 5% (Newman-Keuls, ± standard error); *On MS medium with sucrose (3%) and NAA (3 µM) + BA (6 µM); The data represents the mean of 5 replicates; The data collected after 35 days of culture.

conditions and media could alter the pattern of organogenesis in orchids.

The regenerated plantlets were separated from the shoot buds clumps and maintained on optimum regeneration condition for 2-3 passages for culture proliferation and plantlet growth. About 3 cm long plantlets with distinct pseudobulb, 4-5 leaves and 4-5 roots from regeneration medium were taken out from the regeneration medium and subjected to *in vitro* hardening.

Hardening and field trials of the regenerates

The well rooted healthy plantlets were removed from the regeneration medium and transferred into culture vials containing 1/2 strength of MS salt solution supplemented with 2% (w/v) sucrose but devoid of any PGRs. The cultures were then maintained for ~6-7 wk under identical culture conditions and then the hardened plants were transferred to plastic pots with potting mix. The potted plants were maintained in poly house (ca 70% filtered light) and fed with MS liquid salt solution ($1/10^{th}$ strength) weekly for 2-3 wk. The potted plants were left in the normal full day light conditions which were kept for about 7-8 wk before transferring to the wild. During this process plantlets turned deep green and developed pigments. About ~75% of the transplants survived to form fully developed plants after 2 months of potting. Feeding the plantlets with nutrient salt solution has been reported to be beneficial

for the promotion of orchid survival and growth (Deb and Temjensangba, 2005, 2006a, 2007a; Temjensangba and Deb, 2005b; Pongener and Deb, 2009, 2011a, 2011b). In present study, the plantlets in hardening conditions developed new roots which got attached to the support medium with the passage of time and vigorous plantlets growth was observed. The roots attached themselves mostly to the charcoal pieces and moss, which strongly suggest the suitability of these materials for hardening. The transplanted regenerates were observed to be dark green and healthy with emergence of new roots and leaves just after one month's transfer to potting mix. The protocol developed in the present study provides an alternative route for mass multiplication of this important terrestrial orchid using alternative explants source.

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REFERENCES

Abdul Karim, A.G. and Hairani, H. 1990. Leaf culture of some Malaysian orchids. pp. 1-12. In: *Proceedings of International Conference and Exhibition on Orchids and Ornamental Plants.* Kuala Lumpur, Malaysia.

Arenmongla, T. and Deb, C.R. 2012. Germination of immature embryos and multiplication of *Malaxis acuminated*. Don., an endangered therapeutically important orchid, by asymbiotic culture *in vitro*. *Indian Journal of Biotechnology*, **11**: 464-469.

Arumugam, S., Chu, F.H., Wang, S.Y. and Chang, S.T. 2009. *In vitro* plant regeneration from immature leaflets derived callus of *Acacia confuse* Merr. *via* organogenesis. *Journal of Plant Biochemistry and Biotechnology*, **18**: 197 -201.

Chen, T.Y., Chen, J.T. and Chang, W.C. 2004. Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids. *Plant Cell Tissue and Organ Culture*, **76**: 11-15.

Deb, C.R. and Temjensangba. 2005. *In vitro* regenerative competence of *Cleisostoma racemiferum* (Orchidaceae) aerial roots. *Journal of Plant Biochemistry and Biotechnology*, **14**: 201-204.

Deb, C.R. and Temjensangba. 2006a. On the regeneration potential of *Arachnis labrosa* (Lindl. Ex. Paxt) Reichb. root segments: A study *in vitro*. *Phytomorphology*, **56**: 79-83.

Deb, C.R. and Temjensangba. 2006b. In *vitro* propagation of threatened terrestrial orchid, *Malaxis khasiana* Soland ex. Swartz through immature seed culture. *Indian Journal of Experimental Biology*, **44**: 762-766.

Deb, C.R. and Temjensangba. 2007a. Direct regeneration of shoot buds in *Arachnis labrosa* foliar explants. *Journal of Orchid Society of India*, **21**: 7-9.

Deb, C.R. and Temjensangba. 2007b. Rapid mass multiplication of *Cleisostoma racemiferum* (Lindl.) Garay: An endangered orchid. *Journal of Plant Biology*, **34**: 99-105.

Deb, C.R. and Imchen, T. 2008. *Orchid Diversity of Nagaland*. SciChem. Publishing House, Udaipur, Rajasthan, India.

Deb, C.R. and Sungkumlong. 2010. Regenerative competence of foliar explants of *Coelogyne suaveolens* and *Taenia latifolia*: Two threatened orchids of North-East India. *Applied Biological Research*, **12**: 1-9.

Deb, C.R. and Arenmongla, T. 2011. Morphogenetic induction and organogenic differentiation from foliar explants of *Strobilanthes flaccidifolius* Nees: A natural dye yielding plant. *Journal of Experimental Sciences*, **2**(10): 22-28.

Deb, C.R., Deb, MS, Jamir, NS and Imchen, T. 2009. Orchids in indigenous system of medicine in Nagaland, India. *Pleione*, **3**: 209-211.

Gopi, C., and Ponmurugan, P. 2006. Somatic embryogenesis and plant regeneration from leaf callus of *Ocimum basilicum* L. *Journal of Biotechnology*, **126:** 260-264.

Lakshmanan, P., Geijskes, R.J., Wang, L., Elliott, A., Grof, C.P.L., Berding, N. and Smith, G.R. 2006. Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis

in sugarcane (*Saccharum* spp. inter-specific hybrid) leaf culture. *Plant Cell Report,* **25**: 1007 - 1015.

Li, Z. and Xu, L. 2009. *In vitro* propagation of white-flowered mutant of *Rhynchostylis gigantean* (Lindl.) Ridl. through immature seed-derived protocorm-like bodies. *Journal of Horticulture and Forestry*, **1**: 93-97.

Mathews, V.H. and Rao, P.S. 1985. *In vitro* culture of Vanda hybrid (Vanda TMA X Vanda Miss. Joaquim) 1. Studies on protocorm explants. *Proceedings of Indian National Science Academy (Plant Science),* **51**: 96-103.

Murashige, T. 1974. Plant propagation through tissue culture. *Annual Review of Plant Physiology,* **25**: 135-166.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum*, **15**: 473-497.

Nayak, N.R., Patnaik, S. and Rath, S.P. 1997. Direct shoot regeneration from foliar explants of an epiphytic orchid, *Acampe praemorsa* (Roxb.) Blatter & Mc Cann. *Plant Cell Report*, **16**: 583-586.

Pongener, A. and Deb, C.R. 2009. Asymbiotic culture of immature embryos, mass multiplication of *Cymbidium iridioides* D. Don and the role of different factors. *International Journal of Pharma and Bio Sciences*, **1:**1-14.

Pongener, A. and Deb, C.R. 2011a. *In vitro* mass multiplication of *Cymbidium aloifolium* using immature embryos as explant. *Journal of Plant Biochemistry and Biotechnology*, **20**: 90-95.

Pongener, A. and Deb, C.R. 2011b. *In vitro* regeneration of plantlets of *Cymbidium irridioides* D. Don using nodal segments as explants. *International Journal of Applied Biotechnology and Biochemistry*, **1**: 389-400.

Seeni, S. and Latha, P.G. 1992. Foliar regeneration of endangered red vanda *Renanthera imschootiana* Rolfe (Orchidaceae). *Plant Cell Tissue and Organ Culture*, **29**: 167-172.

Sinha, S.K. and Hegde, S.N. 1999. Regeneration of plantlets from *in vitro* leaf culture of Renades Arunoday Hybrid (*Aerides rosea* Loddiges Ex. Paxt. X *Renanthera imschootiana* Rolfe). *Journal of Orchid Society of India,* **13**: 19-24.

Temjensangba and Deb, CR. 2005a. Regeneration of plantlets from *in vitro* raised leaf explants of *Cleisostoma racemiferum* Lindl. *Indian Journal of Experimental Biology*, **43**: 377-381.

Temjensangba and Deb, C.R. 2005b. Regeneration and mass multiplication of *Arachnis labrosa* (Lindl.ex Paxt.) Reichb: A rare and threatened orchid. *Current Science*, **88**: 1966-1969.

Vij, S.P. 2002. Orchids and tissue culture: Current status. pp. 211-216. **In:** Role of plant tissue culture in biodiversity conservation and economic development (eds. S.K. Nandi, L.M.S. Palni and A. Kumar). Gyanodaya Prakash, Nainital, India.

Vij, S.P. and Aggarwal, S. 2003. Regenerative competence of foliar explants: *Vanda Coerulea* Griff. *Journal Orchid Society of India*, **17**: 73- 78.

Wimber, D.E. 1965. Additional observations on clonal multiplication of *Cymbidium* through culture of shoot meristems. *Cymbidium Society News,* **20**: 7-10.