NBU Journal of
Plant Sciences

Vol. 8, No. 1 March 2014 p. 65-70
ISSN No 09749007

Mass Propagation of an epiphytic orchid *Acampe papillosa* (Lindl.) through *in vitro* seed germination

Subhas Chandra Roy

Plant Genetics & Tissue Culture Laboratory, Department of Botany, University of North Bengal, PO- NBU, Siliguri-734013, WB, India.

Abstract

A method of *in vitro* propagation of a medicinally important epiphytic orchid of NBU campus *Acampe papillosa* has been developed. Three different categories of media (1/2 MS, MS and Knudson C) were used for *in vitro* asymbiotic seed germination for conservation and commercial uses. Seed germination was observed in all the media without any growth supplement or additives although not efficiently. Seeds showed positive germination response in all the three media but the frequency and onset of germination, protocorm formation and associated morphogenic changes leading to plantlets establishment varied with the nature of additional growth stimulus. Different concentrations and combinations of auxin and cytokinins were used to enhance the germination and seedling development. The different concentration of coconut water (CW) was used as additive supplement in this study and 15% CW showed good germination within 22 days in KnC medium. Various concentrations and combinations of plant growth regulators – BAP (1mg l^{-1} to 4 mg l^{-1}), NAA (0.5 mg l^{-1} to 2 mg l^{-1}) and IBA (0.5 mg l^{-1} to 2 mg l^{-1}) were added into the three categories of media as growth stimulants. The induction of PLBs was observed in the absence of any exogenous supply of PGRs, but the addition of BAP (4 mg l^{-1}) with KnC medium improved the shoot growth significantly and enhanced the PLBs development (95% efficiency) with highest shoot length (2.5 cm) after six months of culture. Minimum number of days was required for seedling development (148 days) when cultured into the KnC medium supplement with combinations of BAP (4 mg l^{-1}) and IBA (2 mg l^{-1}). Addition of small quantity of NAA (0.5 mg l^{-1} to 2 mg l^{-1}) was not improved the PLBs or seedling development in compared to BAP and IBA. Rooting was improved by addition of IBA (0.5 mg l^{-1} to 2 mg l^{-1}) into the media. High rate of rooting efficiency was recorded when combinations of auxin and cytokinin was used. Frequency of rooting (95%) was studied in KnC medium containing BAP (4 mg l^{-1}) and IBA (2 mg l^{-1}). Seedling of 2.5 cm long with 3-4 leaves were transferred to earthen pot containing wood charcoal and coco peat for acclimatization under green house conditions. Hardened plantlets showed significantly high survival rate (81%) after seven months of transfer. Therefore, the present method of micro-propagation could be used successfully to propagate this important orchid for commercial production and conservation purposes.

Key-words: *In vitro* propagation, seed germination, Protocorm, *Acampe papillosa* orchid.

INTRODUCTION

Orchid has extremely high floricultural appeal because of their extraordinarily beautiful and highly enchanting flowers with incredible range of variation in floral shape, size, coloration and fragrance belonging to family Orchidaceae (Nongdam and Nirmala, 2012). Their distribution is found around the globe except the freezing Antarctic region and deadly hot desert areas (Sazak and Ozdener, 2006). The family Orchidaceae is probably the largest among all angiosperms and considered as most advanced flowering plants, with an estimated 25000 species (Chugh *et al.*, 2009). About 70% of orchid species are epiphytic that mostly inhabit in the tropics and near about 163 genera and 1100 species have been recorded in India. Among these 300 species are endemic. The North-eastern region of India (which is amongst the 8 hottest biodiversity hotspots of the world) harbors around 876 orchid species constituting nearly 70% of the total orchid flora of India (Medhi and Chakrabarti, 2009) remaining are found in the region of the Eastern and the Western Himalayas. Apart from their ornamental aesthetic value, a large number of species are also medicinally important (Handa, 1986; Piri *et al.*, 2013). The beautiful campus of the University of North Bengal having small forest vegetation with orchid biodiversity and lies between Latitude of 26° 84A North and Longitude of 88°

Corresponding author:
E-mail: subhascr@rediffmail.com

44A East, near Siliguri, Dist- Darjeeling, WB, India, total area 300 acres. The area is seasonally dry and water logged, which generally falls under sub-tropical climatic zone. The average rainfall ranges from 100-300 cm with relative humidity at 80-90% during the rainy season accompanied with varied temperature of 20°C to 38°C during the summer and during winter temperature ranges from 7°C - 20°C. These varying physiographical features coupled with suitable climatic condition of this region offer the best natural habitat for a number of orchid species. It is reported the presence of about 5-10 orchid species under 5 genera in this campus. However, orchid population is reducing at the alarming rate due to extensive collection for illegal orchid trade and habitat destruction. Most orchids show extremely slow rate of vegetative multiplication and propagation through seed germination is also time taking because they need suitable symbiotic association with a mycorrhizal fungus and thus considered very difficult for commercial cultivations. Breakthrough came when it was first established that fungal requirement can be compensated by supply of sugars and other mineral nutrients for *in vitro* asymbiotic artificial orchid seed germination (Knudson, 1922; Arditti *et al.*, 1982). It is still to be learnt about the nutrient requirements of commercially important and/or endangered orchid species due to its large number of genotypic variation. *Acampe papillosa* (Lindl.), is an epiphytic orchid of the NBU campus, has ornamental potential for its evergreen, clustered foliage and multi-flowered, pendulous racemes, with yellow green flowers with anti-rheumatism property of its roots (Bi *et al.*, 2005). Its natural populations are on decline due to commercial collection and habitat destruction.

In the present study an attempt was made to mass propagate this medicinally important epiphytic orchid through *in vitro* asymbiotic seed germination for conservation purposes.

Materials and methods

Surface sterilization of capsule

The green and undehisced capsule of five-month old (Figure 1 A) was harvested from live plants (NBU campus) of *Acampe papillosa* served as source for young seeds with immature embryos. The pod was washed under tap water (30 minutes) followed by treatment with 7.5 % (v/v) Lizol for 30 minutes and surface sterilized for 7 min with $HgCl_2$ solution (0.1%), with 1 to 2 drops of 'teepol' as a wetting agent prior to washing with sterilized distilled water. The pod was also treated with streptomycin (0.03%) for 5 min and also dipped in 1% Bavistin (repeatedly washed with sterilized double distilled water so as to remove all the traces of sterilizing agents), and subsequently, pod was dipped in 70% ethyl alcohol for 30 s, flame sterilized, and was split open longitudinally with a sterilized blade to scoop out the immature embryos, under aseptic conditions and seeds were inoculated into different nutrient media.

In vitro seed germination

The seeds were scoped and transferred on different nutrient media such as ½ MS, MS (Murashige and Skoog, 1962), Knudson C (Knudson, 1922) supplemented with coconut water (10-25% v/v) to study their germination percentage and subsequent seedling development in *A. papillosa*. Different concentration and combinations of auxin and cytokinin (plant growth regulators) such as BAP (0, 1, 2, 4 mgl^{-1}), NAA (0, 0.5, 1, 2 mgl^{-1}) and IBA (0, 1, 2, 3 mgl^{-1}) were added for shooting and rooting. The pH of the culture media was adjusted to 5.6 and was autoclaved at 121° C for 20 minutes under 15 lb $inch^{-2}$ pressures.

Maintenance of culture

The cultures were incubated at 25±2°C under 12 h photoperiod provided by cool white fluorescent tubes Philips (40 µmol m^{-2} s^{-1}). Effect of different growth additives [AC (activated charcoal; 0.2%), YE (yeast extract; 2 g/L.)] was also assessed during the experimentation. Data of different morphogenetic responses were collected after one month intervals up to six months of culture in three replicates. Sub-culturing was done at four week intervals.

Hardening of plantlets

The well-developed seedlings (3-4 cm height) with

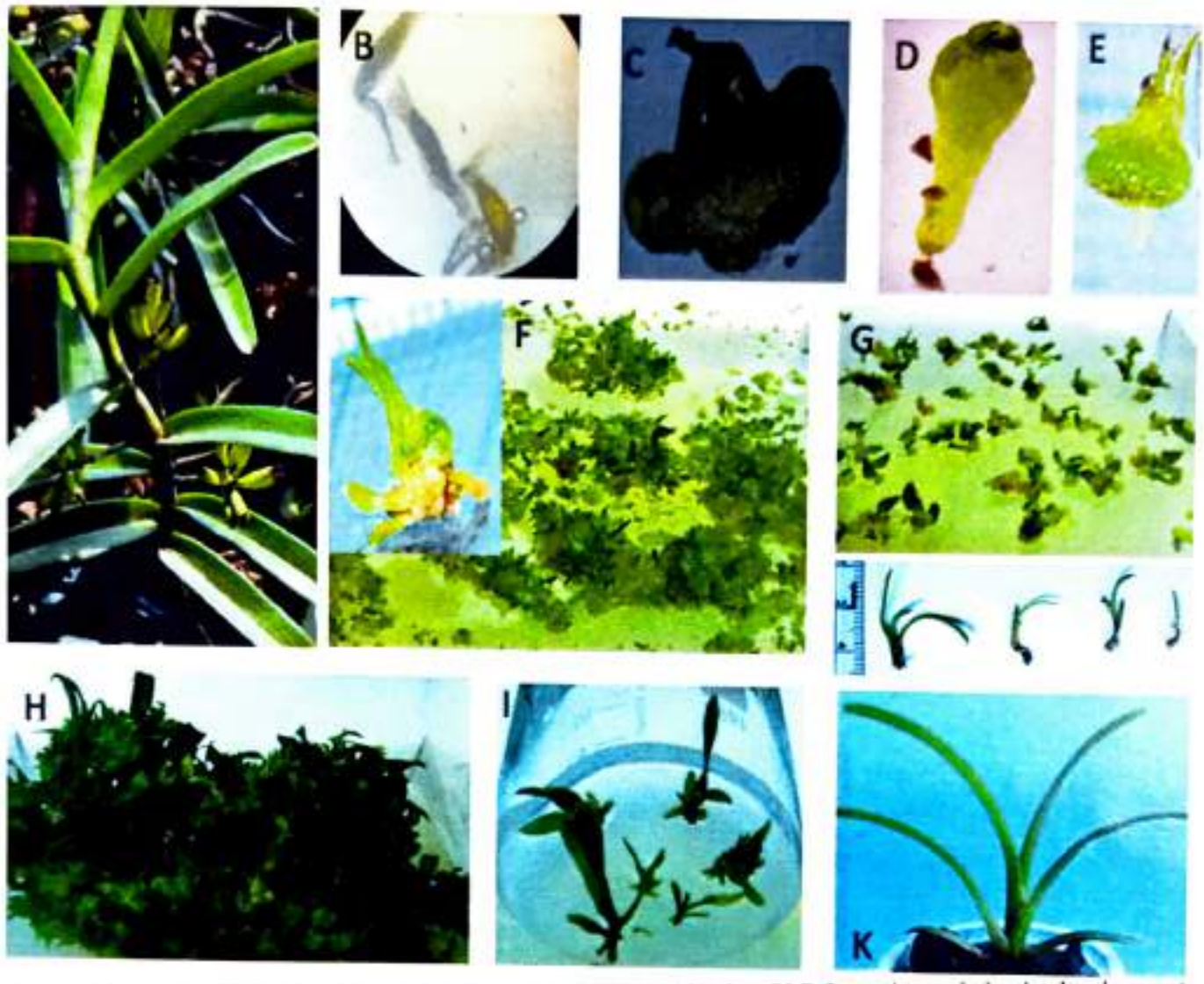

Figure 1. *In vitro* asymbiotic orchid (*Acampe papillosa*) seed germination, PLB formation and plantlet development for mass propagation. (A) Natural habitat of *A. papillosa* in the NBU campus; (B) Immature seeds at the time of inoculation (x40); (C) Vertical section through germinating embryo (x20); (D) Protocorm-like body (PLB) emerged out by rupturing seed coat (x30); (E) Leafy shoot tip and root like structure at the base of a PLB; (F) Multiplication of PLBs (inset enlarge view of PLB); (G) Shoot development from the PLBs; (H) Seedling development and multiplication; (I) Healthy seedlings with root; (K) Plantlet transferred to a pot for hardening under green house conditions.

2 to 3 leaves were removed from culture vessels and thoroughly washed with tap water to remove adhering medium completely without causing damage to the roots. Then plantlets were treated with fungicide solution (Bavistin) at 4% concentration and streptomycin (0.03%) for 5 min. These were then transferred to perforated plastic pots filled with a mixture of uniform, small charcoal pieces, brick pieces, sphagnum moss, and pine bark (1:1:1:1). After a through wash of the pots and the potting mixture in water and treatment with 0.2% diethane M-45 fungicide, the seedling were transplanted. The potted plants were kept under a green house (25% light) and mist irrigated conditions. It showed 70% survival rate of the plantlets. After 2 weeks both misting and foliar application of NPK (17:17:17) were followed.

Results and discussion

Seed germination and PLBs formation

Three categories of media (1/2 MS, MS and KnC) were used to study the efficiency of mass multiplication of an epiphytic orchid of NBU

campus *Acampe papillosa* for conservation purposes and commercial use (Table 1) containing various concentration of additives and plant hormones. Seed germination efficiency was quite high in ½ MS and KnC when supplemented with additives (CW 15%) and plant growth regulators (PGR) - cytokinin and auxin. On set of *in vitro* seed germination was initiated within 22 days in ½ MS and KnC media supplemented with 4 mgl^{-1} BAP in one category and in combination of both 4 mgl^{-1} BAP and 2 mgl^{-1} IBA. In spite of additives (BAP, NAA, IBA, CW) the seed germination rate, PLB formation and plantlets developments was low in MS media. Seed germination started after three weeks of culture initiation and considered to have occurred when the embryo emerged from the ruptured seed testa (Figure 1 B-C). Swelling of the embryos were first noticed within 12 days and maximum 35 days recorded by MS medium while the minimum period 20 days was required by KnC medium supplemented with 4 mgl^{-1} BAP (Table 1). Germination of orchid seeds is not like other seeds because longevity of some orchid seeds is highly variable and may lose their viability in nine months while other in two months or less. Huge numbers of orchid seeds are produced within capsules which are extremely minute in size containing undifferentiated embryo composed of 80-100 cells without any functional endosperm. One of the metabolic machinery (glyoxosome) is not present for conversion of their lapidaceous reserve food material into more utilizable forms (Harrison, 1977) in the seed. Seed requires a symbiotic association with a mycorrhizal fungus in nature (Bernard, 1904). Ever since Knudson (1922, 1925) germinated the seeds of *Cattleya, Laelia* and *Epidendrum* on a sugar rich medium *in vitro*, thus bypassing their fungal requirement. In the present investigation, the seed germination of *Acampe papillosa* was recorded minimum 20 days in KnC medium with plant growth regulator, BAP (4 mg l^{-1}) and maximum 35 days in MS medium. A similar result was observed by other researcher (Hossein *et al.*, 2013) in *Acampe papillosa*. It was reported that minimum 51 to 55 days required for germination of *Dendrobium* sp. in MS medium. Due to non-endospermic nature of the seed, its germination *in vivo* is a unique phenomenon and requires symbiotic fungal association. Different nutrient media were suggested for orchid seed germination and propagation by several authors (Arditti, 1979; Ernst, 1974; Nagaraju and Parthasarathi, 1995; Temjensanba and Chitta 2005; Park *et al.*, 2002). Germinated embryo comes out from the seed coat and form a special structure is called protocorm and then develops into seedling (young plantlet). Protocorm was initially white without any visible shoot apex, but soon they turned green and form leafy primordia at the apex and clusters of rhizoids from the basal parts (Figure 1 D-E) and ultimately form complete seedling with shoot and root. These stages are common to most orchid species (Arditti, 1967). PLB formation during *in vitro* germination is a general phenomenon in the family orchidaceae. They are actually somatic embryo and originate from a single cell (Chang and Chang, 1998).

PLBs (protocorm like body) are formed during seed germination which is a rhizome like small globular structure (Figure 1, D). High rate of PLB formation was recorded in KnC medium supplemented with 4 mgl^{-1} BAP after 56 days of culture inoculation (Table 1). Protocorm formation was observed in the three categories of media (1/2 MS, MS and KnC) with different concentration of CW, BAP, NAA, and combinations of BAP and IBA (Table 1). Highest protocorm like bodies was produced on the KnC medium containing 4 mgl^{-1} BAP, followed by a combination of 4 mgl^{-1} BAP and 2 mgl^{-1} IBA, which also showed multiple shoot formation (Figure 1 G-H). Lowest number of PLBs was produced on the MS medium contained 1mg l^{-1} NAA (Table 2).

Root formation was noted following transfer of the PLBs to the media containing rooting plant hormone (NAA, IBA). Highest frequency of rooting was recorded on the KnC medium supplemented with both 4 mgl^{-1} BAP and 2 mgl^{-1} IBA followed by 4 mgl^{-1} BAP (Table 2). Generally, auxins stimulate root formation and cytokinins enhance shoot development and cell division. In the present study, it was observed that orchid seedlings had significantly higher growth in presence of auxin and cytokinin combinations. Multiplicatiom of protocorms was observed in CW supplemented

Table 1. Effect of different nutrient media on onset of seed germination, PLBs formation and seedling development during asymbiotic orchid seed culture of *Acampe papillosa*

Treatment (Supplements)	Nutrient media								
	½ MS			MS			KnC		
	Onset germination (days)	PLBs (days)	Seedlings (days)	Onset germination (days)	PLBs (days)	Seedlings (days)	Onset germination (days)	PLBs (days)	Seedlings (days)
Control	30 ± 1.04	70 ± 0.97	174 ± 1.78	35 ± 1.02	70 ± 2.8	176 ± 1.99	26 ± 2.09	65 ± 1.07	165 ± 1.12
CW (15% v/v)	24 ± 2.01	62 ± 0.7	157 ± 1.09	26 ± 2.12	66 ± 1.31	168 ± 1.11	22 ± 1.32	62 ± 2.01	155 ± 0.57
BAP (4 mgl^{-1})	22 ± 1.99	58 ± 1.21	151 ± 1.06	24 ± 2.08	62 ± 0.12	163 ± 2.07	20 ± 1.45	56 ± 2.7	150 ± 1.11
NAA (2 mg l^{-1})	25 ± 1.22	60 ± 1.63	161 ± 2.09	25 ± 1.89	63 ± 1.22	165 ± 1.91	24 ± 2.21	64 ± 1.23	157 ± 2.01
IBA (2 mg l^{-1})	25 ± 1.33	59 ± 2.00	158 ± 1.65	24 ± 1.99	63 ± 0.98	154 ± 1.28	24 ± 0.23	65 ± 1.54	157 ± 1.81
BAP (4 mgl^{-1}) + IBA (2 mgl^{-1})	22 ± 0.34	55 ± 1.55	146 ± 0.78	24 ± 1.22	57 ± 1.37	153 ± 0.55	22 ± 1.65	60 ± 1.99	148 ± 1.71

Mean ± SD [5 samples in three replicates]

Table 2. Effects of different media and additives on PLB formation frequency, shoot length (after six months) and root initiation in *Acampe papillosa*

Treatment (Supplements)	Nutrient media								
	½ MS			MS			KnC		
	Frequency of PLB formation (%)	Shoot length (cm) 6 months	Frequency of rooting	Frequency of PLB formation (%)	Shoot length (cm) 6 months	Frequency of rooting	Frequency of PLB formation (%)	Shoot length (cm) 6 months	Frequency of rooting
Control	50	1.5	30	45	1.5	30	50	1.3	40
CW(15% v/v)	70	2.0	50	50	1.2	45	72	1.5	62
BAP (4 mgl^{-1})	90	2.4	50	58	1.4	45	95	2.5	62
NAA (2 mg l^{-1})	37	1.5	70	35	1.2	35	42	1.7	75
IBA (2 mg l^{-1})	35	1.3	90	30	1.3	40	50	2.0	92
BAP (4 mgl^{-1}) + IBA (2 mgl^{-1})	85	2.5	80	50	1.3	45	88	2.6	95

Mean ± SD [5 samples in three replicates]

cultures which favoured germination in *Cattleya, Cymbidium, Rhynchostylis retusa, Dendrobium* and *Paphiopedilum purpuratum* (Chung *et al.*, 1985; Chang *et al.*, 2005).

Plantlets development and acclimatization

PLBs were frequently sub-cultured in different media supplemented with 1- 4 mgl^{-1} BAP for plantlets developments. After 2 months shoot development was observed protruding from the PLBs (Figure 1E-F). Upon sub-culturing into fresh medium, plantlets with well-formed leaves and roots were obtained after 6 months (Figure 1 I-J). Plantlets were transferred into earthen pot containing wood charcoal and coco peat and acclimatized under green house conditions according to Maitra *et al.* (2009). Hardened plantlets showed significantly high survival rate (81%) after seven months of transfer (Figure 1K). The experimental results revealed that KnC medium supplemented with coconut water (CW 15%) and BAP (4 mg l^{-1}) influenced asymbiotic seed germination and PLB formation. It was noticed that induction of PLB could occur in the

absence of any exogenous supply of plant growth regulator, addition of BAP improved the PLB formation and multiplication considerably and IBA enhanced root initiation significantly. The *in vitro* raised plantlets were successfully transferred to potting medium and hardened in the green house conditions with high survival efficiency (81%). The present investigation opens up the procedures for *in vitro* mass propagation of ornamentally and medicinally important orchid *Acampe papillosa* of NBU campus for conservation as well as commercial purposes.

References:

Arditti J (1967) Factors affecting the germination of orchid seeds. Bot Res 33: 1-97

Arditti J (1979) Aspects of the physiology of orccchid. Adv Bot Res 7: 421-655

Bernard N (1904) Recherches experimentales sur les orchidees. Rev Gen Bot 16: 405-451

Chang C, Chen Y C and Yen H F (2005) Protocorm or rhizome The morphology of seed germination in *Cymbidium dayanum* Reichb. Bot Bull Acad Sin 46:71-74

Chung J D, Chun C K, Choi S O (1985) Asymbiotic germination of Cymbidium ensifloium II: Effect of several supplements to the medium. pH values, and light and dark/or dark culture periods on rhizome growth and organogenesis from rhizome. J Korean Soc Hortic Sci 2:186-192

Chang C and Chang W C (1998) Plant regeneration from callus culture of Cymbidium ensifolium var. misericors. Plant Cell Rep 17: 251-255

Ernst R (1974) The use of activated charcoal in asymbiotic seedling culture of Paphiopedilum. Am Orch Soc Bull 43: 3538

Handa S S (1986) Orchids for drugs and chemicals. In: Vij SP (ed.) Biology, conservation and culture of orchids. Affiliated East-West press, New Delhi, pp. 89-100

Hossein Piri, Promila Pathak and Bhanwra R K (2013) Asymbiotic germination of immature embryos of a medicinally important epiphytic orchid *Acampe papillosa* (Lindl.). Afri J Biotech 12: 162-167

Hossain M M, Sharma M, Pathak P (2009) Cost effective protocol for *in vitro* mass propagation of *Cymbidium aloifolium* (L.) Sw.- A medicinally important orchid. Eng Life Sci 9: 444-453

Jena S, Jena Ram Chandra and Rasmita Bhol (2013) In vitro propagation of Acampe papillosa Lindl. (Orchidaceae) through direct somatic embryogenesis using leaf explants. Asi J Biol L Sc 2: 234-240

Knudson L (1922) Non-symbiotic germination of orchid seeds. Bot Gaz 73: 1-25

Knudson L (1925) Physiological study of the asymbiotic germination of orchid seeds. Bot Gaz 79: 345-379

Maitra K, Roy S C and De Sarker D (2009) Mass propagation of *Cymbidium aloifolium* (L.) Sw. from asymbiotically germinated seeds using filter-paper bridge technique. Journal Plant Biol 36 (1&2): 17-22

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Nagaraja V and Parthasarathi V A (1995) Micropropagation of Phaius by seed and protocorm. Ann Plant Physiol 9: 105-108

Park S Y and Chakraborty D (2002) An efficient direct induction of protocorm-like bodies from leaf subepidermal cells of Doritaenopsis hybrid using thin section culture. Plant Cell Rep 21: 46-51

Temjensangba S and Chitta R D (2005) Regeneration and mass multiplication of Arachnis labrosa (Lindle. Ex Paxt) Reichb. A rare and threatened orchid. Curr Sci 88: 1966-1969