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Regeneration and mass multiplication of *Arachnis labrosa* (Lindl. ex Paxt.) Reichb: A rare and threatened orchid

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Asymbiotic seed germination of *Arachnis labrosa* was achieved by culturing immature embryos/seeds 16–18 weeks after pollination. Better germination was recorded on medium enriched with sucrose (3%), coconut water (15%) and α-naphthaleneacetic acid (NAA) and N^6-benzyl adenine (BA) (20.0 + 16.0 μM in combination). After 23–25 days of culture, ~81% germination was recorded on Mitra *et al.* medium followed by Murashige and Skoog (66%), and Knudson 'C' (55%). The protocorm-like bodies differentiated into multiple shoot buds after 20 days of culture on regeneration medium containing sucrose (3%), coconut water (15%), and NAA and BA (10.0 and 8.0 μM respectively, in combination). After maintaining for 10–12 weeks on regeneration medium, the rooted plants were transferred to potting mix and acclimatized before transfer to natural habitat.

ASYMBIOTIC seed germination has emerged as an important tool for propagating a large number of orchid species and hybrids[1]. The orchid seed can also germinate *in vitro* prior to reaching maturity. The technique is variously referred to as ovule/embryo/green pod/green fruit culture[2], which ensures better germination frequency and favours the production of virus-free seedlings at a faster rate. The medium used for asymbiotic germination is more complex than that for symbiotic germination, as all organic and inorganic nutrients and sugars must be in a form readily available to the orchid, without the intermediary fungus[3]. Wimber[4] first formulated and described the shoot-tip-based procedure for mass and rapid clonal propagation of *Cymbidium* species. Incidentally, orchid has been the first floricultural crop to be successfully propagated through shoot-tip culture[5]. This novel technique has generated tremendous interest among orchid growers and revolutionized orchid-based industries world over. *Arachnis labrosa* is a monopodial epiphytic orchid of the Vandaceous group. The flower is pale yellow with irregular dark brown marking and is also called spider orchid. In India, the distribution of this orchid is restricted to few patches in Arunachal Pradesh and Nagaland[6]. In this communication we report the establishment of a feasible protocol of *in vitro* regeneration of plantlets and mass multiplication of *A. labrosa* by green pod culture.

A. labrosa cultures were initiated using immature embryos from green pods in different developmental stages (8–20 weeks after pollination) (WAP) at two week intervals. Seeds were scooped out from sterilized pods and cultured on different media like Knudson C[7], Mitra *et al.*[8] and Murashige and Skoog (MS)[9] containing (NAA) (0–30 μM) (BA) (0–24 μM) singly or in combination (Table 1), sucrose (0–3%; w/v) as organic carbon source and coconut water (0–15%; v/v). Before autoclaving at 121°C and 1.05 kg cm^{-2} for 20 min, pH of the media was adjusted to 5.6 using 0.1 N NaOH and HCl. Cultures were maintained at 25 ± 2°C under cool white fluorescent light at 40 μmol m^{-2} s^{-1} and 12/12 h photoperiod. Cultures were sub-cultured at 4–5 week intervals unless mentioned otherwise. Experiments were repeated at least thrice.

Protocorm-like bodies (PLBs) developed from the cultured immature embryos were maintained on the same basal initiation medium for further development and differentiation. For regeneration and culture multiplication, the differentiated PLBs were separated and cultured on different basal media containing various levels of IAA (0–33 μM), NAA (0–30 μM), BA (0–24 μM) and Kn (0–27 μM) singly or in combination, in conjunction with sucrose (3%) and coconut water (15%).

The tiny plantlets measuring 3–4 cm were transferred to perforated plastic pots of 10 cm diameter with potting mixture containing charcoal pieces, brick pieces, coconut

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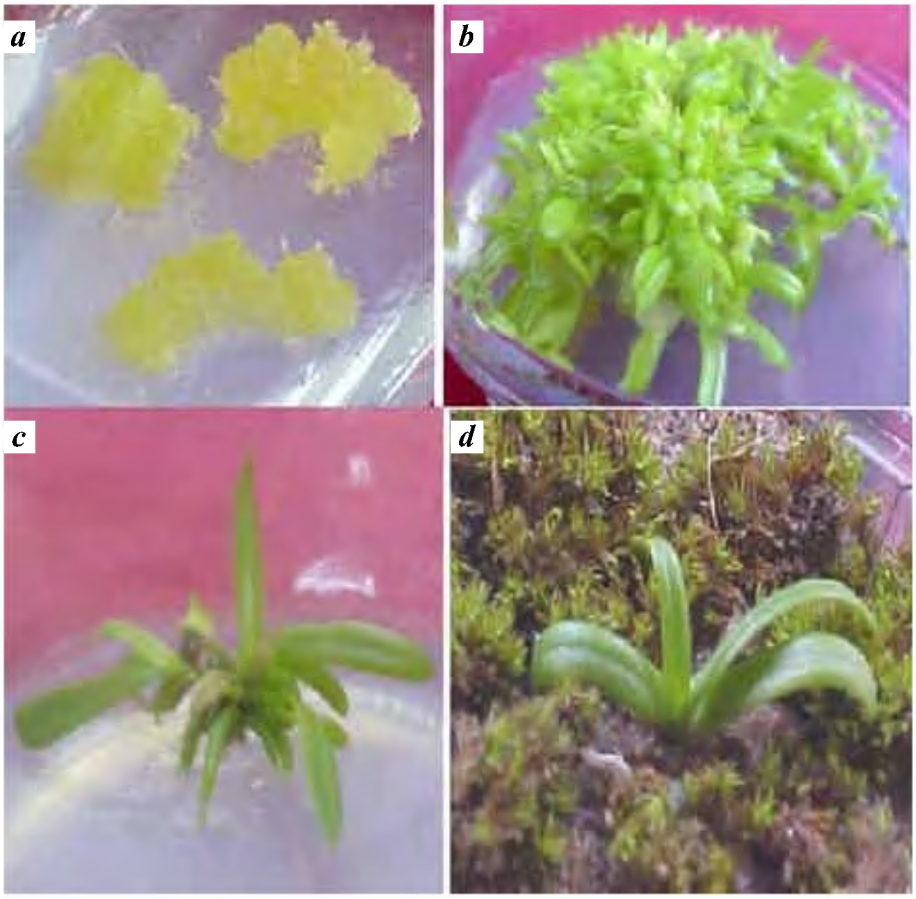

Figure 1. Different stages of immature seed germination and regeneration of plants of *Arachnis labrosa* on Mitra *et al.* medium. ***a***, Initial stage of germination showing greening of cultures; ***b***, PLBs and young plants; ***c***, Multiple shoots/bud formation on regeneration medium; and ***d***, Potted plant.

Table 1. Effect of plant growth regulators (NAA and BA) on immature embryo germination *of Arachnis labrosa**

Growth regulators (μM)		Days taken to germinate	Type of response in subsequent cultures
NAA	BA		
0	0	–	–
10.0	16.0	27	Few green PLBs formed
10.0	24.0	23	Few green PLBs formed
20.0	08.0	23	Few green PLBs formed
20.0	16.0	25	Healthy and green PLBs formed
20.0	24.0	24	Green PLBs formed
30.0	08.0	24	Green PLBs formed
30.0	16.0	25	Few green PLBs formed
30.0	24.0	26	Green PLBs formed

*On Mitra *et al.* medium containing sucrose (3%), coconut water (15%), with green pod age being 16–18 WAP.

husk and decayed wood powder (1 : 1 : 1 : 1), with a layer of moss. Potted plantlets were kept in the polyhouse for hardening. Plantlets were fed with 1/10th MS nutrient salt solution every week for two months.

Among the three different basal media formulations studied, the Mitra *et al.* medium supported higher rate of germination (~ 81%) followed by MS and Knudson C media. Organic carbon source too showed similar variation, as observed in the present investigation. All the media supplemented by sucrose (3%) and coconut water (15%) supported better germination (Table 2). In addition, physiological age of green pod/capsule played an important role on embryo culture. Seed pod age of 8–12 WAP showed no sign of germination, whereas better seed germination was registered from seed pods of 16–18 WAP (Table 3). Seeds collected from green pods of 20 WAP did not support PLB formation though greening of seeds were observed in the early stage of the culture. After 23–25 days of culture, yellowish swelling embryos starts turning green (development of chlorophyll; Figure 1 *a*), the proembryonate structure differentiated and developed a more or less pear-shape PLB within a month, with numerous tiny basal hairs (Figure 1 *b*).

In the present study Mitra *et al.* medium containing NAA + BA (20.0 + 16.0 μM in combination) was found to be the most suitable, which supported higher rate of seed germination. Incorporation of coconut water to basal medium induces and enhances early differentiation of PLBs. Earlier, Talukdar[10] reported similar observation in *Dendrobium*

aphyllum. Leetham[11] reported that a plant growth hormone like cytokinin is present in coconut water. Several authors have reported the importance of seed age[12–14]. Jamir *et al.*[12] reported that *Cymbidium iridioides* seeds better germinated when cultured from 120-day-old green pod. The earliest stage at which the embryos can be cultured successfully varies within the orchid genotype and the local conditions[15]. It is interesting to note that younger embryos germinate better than the older ones, since they may lack dormancy or inhibitory factors[16].

Table 2. Effect of basal medium, sucrose and coconut water on immature embryo germination of *Arachnis labrosa*

Basal medium	Sucrose conc. (%)	Coconut water conc. (%)	Response (% germination) (M ± SD)*
Knudson C	2	10	35.40 (± 0.5)
	2	15	55.50 (± 1.0)
	3	5	40.00 (± 0.5)
	3	10	44.00 (± 1.0)
	3	15	44.00 (± 0.5)
Mitra *et al.*	1	15	20.00 (± 0.5)
	2	5	–
	2	10	40.50 (± 1.5)
	2	15	66.60 (± 1.0)
	3	5	55.50 (± 1.5)
	3	10	70.00 (± 1.5)
	3	15	81.25 (± 1.0)
MS	2	15	60.00 (± 1.0)
	3	5	55.50 (± 1.5)
	3	10	66.60 (± 1.5)
	3	15	66.60 (± 1.0)

*M ± SD, Mean ± standard deviation.

Table 3. Effect of age of green pod/capsule on immature embryo culture of *A. labrosa*

Medium*	Age of capsule (WAP)	Average time taken (days) for PLBs formation
Knudson C	8	–
	10	–
	12	–
	14	–
	16	30
	18	28
	20	–
Mitra *et al.*	8	–
	10	–
	12	45
	14	26
	16	27
	18	27
	20	–
MS	8	–
	10	–
	12	–
	14	27
	16	27
	18	25
	20	–

*Medium containing sucrose (3%), coconut water (15%) and NAA + BA (20.0 + 16.0 µM in combination).

PLBs developed from the germinating seeds after 52–60 days of culture were allowed to differentiate in the same germinating medium. The pear-shaped PLBs with tiny leaf sprouting were developed after nearly 25–30 days of germination. The highest rate of germination and better development of PLBs were obtained on Mitra *et al.* medium containing NAA + BA (20.0 + 16.0 µM) in combination (Table 1). Upon sub-culturing, PLBs differentiated into shoot and roots.

Upon culturing on the medium supplemented with NAA + BA (10.0 + 8.0 µM respectively, in combination), sucrose (3%) and CW (15%) developed multiple shoot buds and PLBs (data not presented). As many as 20 shoot buds/PLBs formed after 20 days of culture on MS medium. The PLBs and young plantlets converted into well-rooted plantlets within 8–10 weeks, when they were maintained on regeneration medium (Figure 1 *c*). After maintaining for 2–3 passages on the regeneration medium the plants were taken out, washed thoroughly to remove traces of agar and transplanted to community pots containing different potting mix (Figure 1 *d*). About 60% of the potted plants survived after two months in the polyhouse. The transplanted plants were acclimatized in the polyhouse for 2–3 months and transferred to the wild.

The present investigation opens up the route for *in vitro* clonal mass multiplication of this extremely rare and threatened species, which will ensure the continued presence of this elegant orchid in nature. Further work on *in vitro* conservation strategies of *A. labrosa* is in progress.

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Deep-sea benthic foraminifera from gas hydrate-rich zone, Blake Ridge, Northwest Atlantic (ODP Hole 997A)

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Certain species of benthic foraminifera feed on rich bacterial food sources at methane seeps, indicating their potential as proxy for methane fluxes in the geological record. Several of these species have been reported in different methane-rich marine settings and have proved to be good indicators of methane eruptions. The Blake Ridge, located ~350 km off the coast of South Carolina, northwestern Atlantic, is a large drift deposit and a proven gas hydrate field, as is evident by the geochemical anomalies and presence of a bottom simulating reflector. This area thus offers good opportunity to analyse benthic faunal–gas hydrate relationship over different timescales. Our newly generated benthic foraminiferal faunal and published total organic carbon data from Ocean Drilling Program Hole 997A suggest *in situ* production of methane by bacterial decomposition of organic matter. We suggest that the fluctuating sea level in response to changes in the Northern Hemisphere continental ice volume may have caused the release of methane from the Blake Ridge gas hydrates during the past 3 Ma. We expect the results of this study to help in the exploration of gas hydrates on the continental shelf of India, which archives a thick pile of sediments with high organic carbon content – ideal for gas hydrate formation.

BENTHIC foraminifera are an important component of the marine community and are sensitive to environmental changes. Over the last three decades, scientists have increased their interest to understand different aspects of benthic foraminifera for palaeoenvironmental reconstructions. The wide geographical and bathymetric distribution, high sensitivity to various ecological factors, extensive morphological diversity, and well-preserved fossil record make them an important tool in palaeoceanography and palaeoclimatology. Some species of benthic foraminifera have been found associated with rich organic carbon content of marine sediments[1–6]. Benthic foraminifera have been found in different methane-rich marine settings and have proved to be good indicators of methane releases[7–11]. Some species prefer to feed on rich bacterial food sources at methane seeps, showing their potential as indicators of methane release in the geological record[11]. Some methane-loving taxa include species of *Uvigerina*, *Bolivina*, *Bulimina*, *Chilostomella*, *Globobulimina* and *Nonionella*[7–13], which can withstand such stressful conditions. Highly depleted carbon isotopic values in the shells of dead or living benthic and planktic foraminifera and other proxies help in identifying the methane-rich environment. Very high negative excursion (–6 to –2‰) of $\delta^{13}C$ in the shells of *Uvigerina*, *Bolivina* and *Nonionella* have been found related to methane excursions[8–10,13]. In the present study, we have attempted to understand the relation between deep-sea benthic foraminifera, *in situ* methane formation and methane releases from Ocean Drilling Program (ODP) Hole 997A, Leg 164, Blake Ridge, northwest Atlantic during the latest Miocene–Pleistocene (Figure 1). We propose to test our model based on benthic foraminifera in the marine sediments of the Indian Ocean in identifying gas hydrate horizons in future endeavours.

ODP Hole 997A is located on the crest of the Blake Outer Ridge (31°50.588′N; 75°28.118′W; water depth 2770.1 m), northwest Atlantic, off the east coast of United States of America[14] (Figure 1). Geophysical survey shows a strong bottom simulating reflector (BSR) present between the gas hydrate zone (top) and free gas zone (below), indicating that the supply rate of methane in Hole 997A exceeds the critical value[15]. Using other geochemical and geophysical proxies (temperature, interstitial water, chloride content and electrical resistivity data), it is proved that disseminated gas hydrate occurs throughout the sedimentary section between ~180 and ~450 m below seafloor (mbsf)[14], which may extend up to ~30 mbsf[16] (Figure 2). Hole 997A consists of a pile of Neogene drift deposits dominated by fine-grained nannofossil-bearing hemipelagic sediments[17] accumulated at unusually high rates during the late Miocene (average 7.97 cm/kyr) and Pliocene (average 9.91 cm/kyr), with a substantial drop in sedimentation rate during the Pleistocene (average 4.61 cm/kyr). This hole is presently situated under the profound influence of the northward-flowing Gulf Stream surface current as well as southward-flowing Western Boundary undercurrent, which carried the clastic materials and formed the ridge[18]. The modern lysocline lying between the 4000 and 4350 m water depth is related to the mixing zone of Antarctic Bottom Water and North Atlantic Deep Water in the subtropical northwest Atlantic[19].

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