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An effective nutrient medium for asymbiotic seed germination and large-scale *in vitro* regeneration of *Dendrobium hookerianum*, a threatened orchid of northeast India

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Abstract

Background and aims *Dendrobium hookerianum* is a rare and threatened epiphytic orchid of northeast India. Prospects for conservation would be strengthened by developing an *in vitro* method for mass propagation. Seeds are minute and difficult to use directly in the field for this purpose, being non-endospermous with a low nutrient content and dependent on a specific fungus for germination and early seedling development. Although produced in large numbers (2–3 million per capsule), <5 % germinate naturally in the wild. Our objective was to develop a rapid and successful method for *in vitro* propagation based on an initial *in vitro* asymbiotic seed germination step that achieved high percentages.

Methodology Effects of four different media, i.e. (i) Murashige and Skoog (MS), (ii) Mitra *et al.*, (iii) Knudson (KC) and (iv) Gamborg *et al.* (B_5), were evaluated for large-scale multiplication by asymbiotic seed germination. Seedling leaf number, shoot number, shoot length, root number and root length were scored. After 7–8 months, large numbers of well-rooted plantlets were transferred to a glasshouse in thermocol pots containing compost. Six different composts based on broken brick and charcoal were compared for their ability to support further development over 90 days of hardening.

Principal results The fastest and highest percentage seed germination was achieved using MS medium. Seeds on MS medium germinated in 3–4 weeks compared with 7–8 weeks on B_5 medium. Seedling development was also superior on MS medium. The inclusion of plant growth regulators was unnecessary. Compost comprising broken brick and charcoal with an upper layer of moss was found to be the most suitable for the survival of transferred plantlets. Ninety per cent survival of plantlets was achieved 90 days after transfer to a glasshouse.

Conclusions The use of MS culture medium is well suited for the mass multiplication of *D. hookerianum* plants intended for re-introducing this threatened orchid into the wild.

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Introduction

The northeast region of India harbours ~750–800 species of orchid (Chowdhery 2001). However, this rich diversity, both in the region and in the country as a whole, is under threat for various reasons. These include deforestation, uncontrolled commercial exploitation, illegal export and the practice of slash and burn cultivation that is widespread in northeast India. At present, all the orchids are listed in the International Union of Conservation of Nature and Natural Resouces (IUCN) Red Data Book. The family Orchidaceae is also included in Appendix-II of the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Senthilkumar 2001). *Dendrobium hookerianum* is an epiphytic orchid included in these lists as a rare and threatened species in northeast India. The orchid has ornamental value and is commercially important due to its bright yellow flower, the pigment from which is used by the textile industry (Chowdhery 1998). There is a clear need to conserve this rare, beautiful and useful species from extinction. We believe that it will be possible to increase its population size by using mass *in vitro* seed germination to generate large numbers of plants to re-introduce into the wild (Wochok 1981). However, at present, there is no established means of achieving mass *in vitro* germination and plantlet culture.

The minute orchid seeds are produced profusely (2–3 million per capsule) but are non-endospermous and contain almost no nutrients (Vu Quoc Luan et al. 2006). In nature, germination and early development are therefore reliant upon a highly specialized fungal association (Rao 1977; Hazarika and Sarma 1995). Consequently, <5 % of the seeds germinate in the wild. A method for mass *in vitro* seed germination and propagation will need to circumvent these requirements (Arditti and Ernst 1984; Sharma and Tandon 1987; Kumaria and Tandon 1991; Kondo *et al.* 1997; Gangaprasad *et al.* 1999; Nagaraju *et al.* 2003; Roy *et al.* 2011). The appropriate choice of nutrient medium is crucial in this regard, but it is known that responses of orchid seeds to different nutrient media vary between species.

The technique of asymbiotic seed germination by *in vitro* culture was first introduced by Knudson (1922). Since then, *in vitro* seed germination protocols have been established for many orchid species, and a number of media and salts have been used for germination and propagation (Arditti and Ernst 1993). Orchid seeds and seedlings have often been reported not to require exogenous auxin (Tamanaha *et al.* 1979). In the present study, we describe an efficient method for mass propagation of *D. hookerianum* through asymbiotic seed germination that is intended to underpin *ex situ* conservation.

Materials and methods

Plants of *D. hookerianum* were collected from Upper Shillong, Meghalaya, India, during October–November 2009 and grown in a glasshouse at the Plant Biotechnology Laboratory, Centre for Advance Studies in Botany, North-Eastern Hill University, Shillong, Meghalaya, India. Purplish-green fruit capsules were collected after 8–9 months, washed in tap water followed by sterile distilled water, dipped in 70 % (v/v) alcohol and flamed 3–4 times with a spirit burner. The surface-sterilized capsules were then opened using a sterile surgical blade and seeds removed. Approximately 1 g of seeds was distributed thinly over the surface of 15 mL of four different basal liquid media, i.e. Murashige and Skoog (MS, 1962), Mitra *et al.* (1976), Kundson (KC, 1946) and Gamborg *et al.* (B_5, 1968), in test tubes. The pH was adjusted to 5.8 ± 0.02 using 1 N NaOH before autoclaving at 121 °C for 15 min. Culture tubes were kept in the dark for 2 weeks followed by dim white illumination (60 μmol m^{-2} s^{-1}) for a 12-h photoperiod at 25 ± 2 °C and at 70–75 % relative humidity (RH). There were 10 replicates and the experiment was repeated twice. Percentage seed germination was determined microscopically after 60 days of culture and calculated by using the formula: % seed germination = number of germinated seeds/total number of seeds × 100. At intervals, the development of protocorms was categorized into four stages: (i) embryo swollen, (ii) appearance of protomeristem, (iii) initiation of leaf and (iv) initiation of roots.

After germination, the growth and development of seedlings in different media were also studied. Ninety-day-old seedlings with leaf initials but without roots were cultured on the same media used for germination and monitored for leaf number, shoot number, shoot length, root number and root length. Data were analysed using one-way analysis of variance Fisher's least significant difference (LSD) test at the 5 % level of probability (software Origin 7.0).

Seedlings ~4–5 cm tall and with well-defined roots were obtained after 7–8 months. These were removed from culture vessels and washed thoroughly with tap water, to remove adhering medium without damaging the roots, before transplanting into perforated free-draining plastic pots (10 × 7 cm size) containing different mixtures of compost: broken brick and charcoal (1 : 1); broken brick and charcoal (1 : 1) + a top layer of moss; broken brick and charcoal + decaying litter (1 : 1 : 1); broken brick and charcoal + decaying litter (1 : 1 : 1) + a top layer of moss; broken brick and charcoal + coconut

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Table 1 **Effect of different media on seed germination and time taken for protocorm formation and development (in weeks) of *D. hookerianum*.**

Media	Germination* %	Protocorm developmental stages** (weeks)				Remarks
		I	II	III	IV	
MS	95.27 ± 0.68[a]	3	4	6	10	Protocorms round and dark green; differentiated into healthy shoots and roots
Mitra	87.85 ± 0.81[b]	4	6	8	12	Protocorms small and green; differentiated into shoots and roots
KC	73.00 ± 1.23[c]	4	6	9	13	Protocorms small and yellow; differentiated into unhealthy shoots and few roots with no further growth
B_5	51.38 ± 1.31[d]	7	9			Protocorms small and green; turned brown after certain period

*Data recorded after 60 days of inoculation.
**I, embryo swollen; II, appearance of protomeristem; III, leaf initial stage; IV, root initial stage.
Values are mean ± SE. Means followed by the same letter in the column are not significantly different as indicated by Fisher's LSD ($P = 0.05$).
Means of 10 values were taken and the experiment was repeated twice.

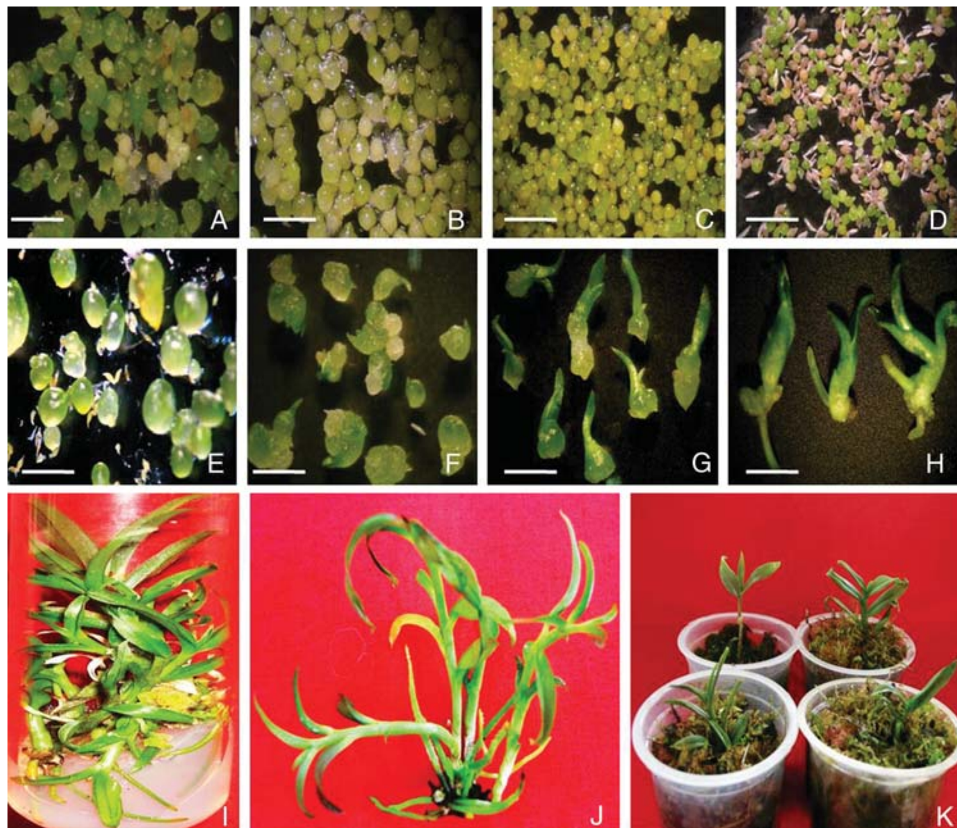

Fig. 1 **Asymbiotic seed germination of *D. hookerianum* on different media after 60 days: (A) MS, (B) Mitra, (C) KC, (D) B_5 (bar = 1 mm).** Different stages of protocorm development on MS medium: (E) embryo swollen, (F) protomeristem, (G) leaf initial, (H) root initials (bar = 1 mm), (I) seedlings on MS medium after 90 days, (J) *in vitro* rooted plantlets from activated charcoal and (K) hardened plants.

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Table 2 Effect of different media on growth and development of *D. hookerianum* seedlings after 90 days of culture.

Medium	Leaf number	Shoot number	Shoot length (cm)	Root number	Root length (cm)
MS	5.3 ± 0.30^{a}	1.4 ± 0.16^{a}	4.22 ± 0.20^{a}	2.6 ± 0.22^{a}	1.60 ± 0.13^{a}
Mitra	3.2 ± 0.24^{b}	1.2 ± 0.13^{ab}	1.73 ± 0.10^{b}	2.1 ± 0.23^{a}	1.13 ± 0.15^{b}
KC	3.1 ± 0.27^{bc}	1.0 ± 0^{b}	0.76 ± 0.03^{c}	2.3 ± 0.15^{a}	0.40 ± 0^{c}

Values are mean ± SE. Means followed by the same letter in the column are not significantly different as indicated by Fisher's LSD ($P = 0.05$).
Means of 10 values were taken and the experiment was repeated twice.

Table 3 Re-establishment of *D. hookerianum* plantlets obtained in MS medium after 90 days of hardening under glasshouse conditions.

Treatment	Survival (%)	Height (cm)
Broken brick + charcoal (1 : 1)	70 ± 4.7	4.6 ± 0.9
Broken brick + charcoal (1 : 1) + layer of moss	90 ± 4.1	5.3 ± 0.9
Broken brick + charcoal + decaying litter (1 : 1 : 1)	55 ± 2.3	4.5 ± 0.9
Broken brick + charcoal + decaying litter (1 : 1 : 1) + layer of moss	59 ± 3.6	4.9 ± 0.5
Broken brick + charcoal + coconut husk (1 : 1 : 1)	30 ± 2.5	2.1 ± 0.6
Broken brick + charcoal + coconut husk (1 : 1 : 1) + layer of moss	37 ± 3.6	2.8 ± 0.6

Values represent means ± SE.
Means of 10 values were taken and the experiment was repeated twice.

husk (1 : 1 : 1); broken brick and charcoal + coconut husk (1 : 1 : 1) + a top layer of moss.

A single plantlet was grown in each pot and each was covered for 2–3 weeks with a pierced polythene bag, sprayed with water to inhibit dehydration and moved to a glasshouse for growing on. The minimum and maximum temperatures were 18 and 25 °C, respectively, and RH was 70–80 %. Plantlets were watered on alternate days and fed with 1/10 strength MS nutrient salt solutions (diluted 10 times) every 2 weeks. Plants were assessed after 90 days.

Results

The speed and extent of seed germination and protocorm development varied with the medium used. Maximum germination percentage and the appearance of protocorms were achieved on MS medium (95.27 ± 0.68), followed by Mitra (87.85 ± 0.81), KC (73.00 ± 1.23) and B_5 (51.38 ± 1.31) (Table 1; Fig. 1A–D). The development of protocorms into seedlings was also superior on MS medium (Fig. 1E and F). On MS medium, the first sign of germination (greening of the embryos) was seen after ~2 weeks. The protocorms began to form 2–3 weeks later. Embryo greening was 1 week slower on Mitra and KC media and 2–3 weeks slower on B_5 medium compared with MS medium. Protocorm formation on B_5 took place at the end of the seventh week, after which further growth was arrested. Leaf number (5.3 ± 0.30), shoot number (1.4 ± 0.16), shoot length (4.22 ± 0.20 cm), root number (2.6 ± 0.22) and root length (1.6 ± 0.13 cm) on MS medium were considerably greater after 90 days of culture (Table 2; Fig. 1I) compared with the other media. Of the various compost mixtures used, the compost made up of broken brick and charcoal with an upper layer of moss was found to be the most suitable for the survival of transferred plantlets. This approach supported 90 % survival after 90 days of hardening under glasshouse conditions (Table 3; Fig. 1K).

Discussion

The choice of culture medium strongly affected germination, presumably because of differences in the balance and supply of organic and inorganic nutrients (Arditti 1982; Arditti and Ernst 1984; Zeigler *et al.* 1985; Van Waes and Debergh 1986). The importance of NH_4^+ and NO_3^- ions (individually or in combination) during *in vitro* germination of orchid seeds as a source of nitrogen is well established (Nongrum *et al.* 2007; Dohling *et al.* 2008). The source of nitrogen in MS medium is ammonium nitrate, whereas in Mitra, KC and B_5 it is in the form of ammonium sulphate (Table 4). Furthermore, NO_3^- in the form of calcium nitrate ($CaNO_3$) is present in Mitra and KC media. Alan (1989) reported that $CaNO_3$ might lower nitrogen content compared with other mineral elements. The presence of ammonium nitrate in MS medium may explain the high germination rate because NH_4^+ is readily assimilated during the initial stages of development and greatly influences

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Table 4 **Inorganic and organic components, and gelling agent of the media.**

Constituents	MS	Mitra	KC	B_5
Inorganic salts ($mg\ L^{-1}$)				
NH_4NO_3	1650.00	—	—	—
KNO_3	1900.00	180.00	180.00	2500.00
KH_2PO_3	170.00		150.00	—
$Ca(NO_3)_2 \cdot 4H_2O$	—	200.00	200.00	—
$MgSO_4 \cdot 7H_2O$	370.00	200.00	250.00	250.00
$(NH_4)_2 \cdot SO_4$	—	100.00	100.00	134.00
$MgCl_2 \cdot 6H_2O$	—	—	—	—
KI	0.83	0.03	80.00	0.75
H_3BO_3	6.20	0.06	6.20	3.00
$MnSO_4 \cdot 4H_2O$	22.30	0.40	0.075	10.00
$ZnSO_4 \cdot 7H_2O$	8.60	0.05	—	2.00
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.05	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.25	0.05	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	—	0.025	0.025
$FeSO_4 \cdot 7H_2O$	27.80	27.80	27.30	27.80
$Na_2EDTA \cdot 2H_2O$	37.30	37.30	37.30	37.30
$CaCl_2 \cdot 2H_2O$	440.00	—	—	150.00
$CO(NO_3)_2 \cdot 6H_2O$	—	0.05	—	—
$NaH_2PO_4 \cdot 2H_2O$	—	250.00	—	150.00
$MnCl_2$	—	—	3.90	—
Organic nutrients ($mg\ L^{-1}$)				
Thiamine HCl	0.10	0.30	0.30	10.00
Nicotinic acid	0.50	1.25	—	—
Pyridoxine HCl	0.50	0.30	0.30	—
Glycine	2.00	—	—	2.00
Riboflavin	—	0.05	0.30	—
Biotin	—	0.50	—	—
Folic acid	—	0.30	—	—
Meso inositol	100.00	—	—	100.00
Sucrose	30 000.00	20 000.00	20 000.00	20 000.00
Gelling agents ($g\ L^{-1}$)				
Agar	8.00	9.00	12.00	8.00
pH	5.8	5.6	5.2	5.8

growth and differentiation (Raghavan and Torrey 1964; Kramer and Kozlowski 1979). The high germination and strong further development in MS medium could be attributed to the fact that MS medium is also especially rich in both macro- and micro-nutrients. The nitrogen source has previously been shown to affect germination of various orchid species (Van Waes and Debergh 1986; Anderson 1996; Stewart and Kane 2006). The poor

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seed germination on KC and B_5 media has also been reported for other orchids (Sharma *et al.* 1991; Dohling *et al.* 2008) despite these media being suitable for *Dendrobium chrysanthum* (Hajong *et al.* 2010). Both the promoting and inhibitory effects of KC medium on the seed germination of many orchid species have been reported by others (Bopaiah and Jorapur 1986; Sharma and Tandon 1987; Pyati and Murthy 1995; Nongrum *et al.* 2007). According to Yam *et al.* (1989), the nutritional requirements of germinating orchid seeds vary due to their physiological state and this may be species specific. In the present study, germination did not require the inclusion of plant growth regulators, suggesting that sufficient endogenous hormones were already present (Lo *et al.* 2001). The nutrient requirement of orchid seeds in terms of quantity as well as form may vary at different stages of development (Arditti and Ernst 1984).

Our plantlets were sub-cultured from time to time in MS medium with activated charcoal (1 g L^{-1}). As is well known, activated charcoal adsorbs phenolic substances released by the plantlets into the media (Fridborg *et al.* 1978; Pan and Van Staden 1998), and stimulates rooting (Van waes 1987; George and Ravishankar 1997) (Fig. 1J). The survival rate of the transferred plantlets in a compost mixture made up of broken brick and charcoal pieces with a covering layer of moss was 90 %. The brick and charcoal provided good drainage and aeration to the roots, and the moss is thought to retain moisture content at an optimal level. In the present study, feeding the plantlets with diluted 1/10 MS nutrient solution was beneficial for growth of the transferred plantlets. This is consistent with the earlier report of Kumaria and Tandon (1994).

Conclusions and forward look

The present study is the first to report a successful and efficient protocol for asymbiotic seed germination and for the regeneration of a large number of plantlets of *D. hookerianum*. For conservation of plants, the seeds are, in general, preferred for propagation because they maintain maximum genetic diversity. *In vitro* seed germination ensures germination in this hard-to-germinate taxon and this enabled us to study morphogenetic changes during seedling development. The mass propagation of orchids through asymbiotic seed germination was achieved and can now be considered as a viable tool in the conservation of the declining native orchid population. It offers a ready means for raising the large numbers of plants needed for *ex situ* conservation of this threatened orchid.

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Contributions by the authors

All authors contributed to a similar extent overall and have seen and agreed to the submitted manuscript.

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Conflicts of interest statement

None declared.

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