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Germination of immature embryos and multiplication of *Malaxis acuminata* D. Don, an endangered therapeutically important orchid, by asymbiotic culture *in vitro*

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The effect of culture conditions and developmental stages of embryos on asymbiotic germination of immature seeds of *Malaxis acuminata* D. Don, an endangered therapeutically important terrestrial orchid, was investigated. Immature seeds of ~7-8 wk after pollination (WAP) were germinated on MS medium containing sucrose (3%, w/v) and α-naphthalene acetic acid (NAA; 4 µ*M*) under diffused light condition (20 µmol m^{-2} s^{-1}), where about 85% seeds germinated after 135 d of culture. The full light condition (40 µmol m^{-2} s^{-1}) did not support healthy germination. The germinated seeds converted into protocorm-like bodies (PLBs) and further differentiated into young plantlets on the same germination medium. The rooted plantlets with well expanded leaves and distinct pseudobulb were achieved on MS medium containing sucrose (3%), activated charcoal (AC; 0.3%, w/v), and NAA and benzyl adenine (BA) (3 µ*M* each in combination), where as many as 18 shoot buds/PLBs developed per subculture. Amongst the three different organic carbon sources and two basal media tested, only sucrose supported the optimum plant growth and culture proliferation when maintained on MS medium. Incorporation of AC (0.3%) in the regeneration medim accelerated the culture proliferation and distinct pseudobulb formation. The hardened plants transferred to potting mix and maintained in the polyhouse (75% shade) before transferring to the wild, where ~75% transplants survived after 2 months of transfer.

Keywords: Activated charcoal treatment, asymbiotic immature embryo culture, *Malaxis acuminata*, micropropagation, therapeutically important orchid

Introduction

The family *Orchidaceae* is one of the largest families in the plant kingdom and represents about 30,000 species of orchids[1]. It is also one of the most fascinating groups of ornamental plants[2]. However, many orchid species are endangered due to environmental disruption, destruction of natural habitats, over exploitation for horticultural and medicinal purposes. *In situ* conservation by conventional means is very difficult because of relatively slow growth of orchids and low germination rates of seeds, which requires symbiotic association with mycorrhizal fungi in natural habitats[2-5]. Thus, in recent years, maintenance of living collections has been considered an important aspect of conservation.

Orchids, besides their ornamental value, are also important therapeutically as they are sources of curare compounds to several ailments. The genus *Malaxis* comprises of about 300 species, which are distributed throughout the tropical to temperate climate region[3]. About 19 species of the genus are represented in India. Many of them, including *Malaxis acuminata* D. Don, are the components of '*Ayurvedic*' drug preparations. The dried pseudobulbs of *M. acuminate* are important ingredient of '*Ashtavarga Drug*' used in the preparation of '*Chyavanprash*', an energetic herbal tonic, and also to cure tuberculosis[1,3,6]. The species grows on decayed forest litter of primary forests. The loss of forest covers due to anthropogenic activities, animal grazing and extensive collection of the rhizomes for drug preparations turned the species to endangered category.

The conventional propagation of the species is too slow and unable to overcome the threat of extinction. The seed germination of terrestrial orchids under natural conditions has been found more difficult than that of epiphytic species[7]. Thus, i*n vitro* propagation strategies are a viable alternative for the rapid propagation of orchids. The *in vitro* germination of orchids are influenced by various factors like light condition[3,5,7-9], developmental stage of green pods/embryos, plant growth regulators (PGRs), etc[3,5].

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In the present study, we have evaluated the effects of illumination, developmental age of immature embryos, addition of PGRs and quantities of sucrose in medium on the asymbiotic germination of *M. acuminata*. We have also described the role of activated charcoal (AC), different basal media, quality and quantity of different organic carbon sources and PGRs on the plantlet regeneration, pseudobulb enlargement and mass multiplication of this species.

Materials and Methods

M. acuminata plants were collected from the primary forest floor of Mokokchung district, Nagaland, India at an altitude of approximately 1400 MSL (Fig. 1). The plants were grown in the experimental garden of Botany Department, Nagaland University, Nagaland at a temperature of ~20/15°C (day/night). The green pods of various developmental stages starting from 4 wk after pollination (WAP) to 12 WAP were harvested at 1 wk interval and used for the present study. Murashige and Skoog (MS) medium[10] gelled with agar (0.8%, w/v) (Hi-Media, India) was used as the basal medium and supplemented with sucrose (0-4%, w/v), α-naphthalene acetic acid (NAA) and benzyl adenine (BA) at concentrations of (0-10 µ*M*) either alone or in combination. The *p*H of the medium was adjusted to 5.6 and about 12 mL medium was dispensed in each borosilicate test tube (25x150 mm^2) (Borosil, India) and cotton plugged. The medium was sterilized by autoclaving at 121°C for 20 min at a pressure of 1.05 Kg cm^{-2}.

The immature embryos/seeds were sterilized and sown as reported by Temjensangba and Deb[11] unless mentioned otherwise. The green pods of various developmental stages were surface sterilized with a solution of $HgCl_2$ (0.2%, w/v) for 5 min and washed 4-5 times with sterilized distilled water. The pods were dipped in ethanol and flamed just before scooping out the seeds. Approximately 200 seeds were sown on the medium in each test tube. For each treatment 20 test tubes were maintained. The inoculated test tubes were incubated at 25±1°C under two light conditions, *i.e.*, normal laboratory light (40 µmol m^{-2} s^{-1}) and 50% diffused light (20 µmol m^{-2} s^{-1}) provided by white fluorescent lamps with 12/12 h photoperiod. The first stages of germination consist of yellowish nodular swellings of embryos, subsequent rapture of the testa and enlargement of the embryo to a top-shaped protocorm-like body (PLB). Germination was considered to have occurred when the embryo emerged from the testa and the size of the embryo doubled. The germination frequencies of seeds/embryos were scored under a light microscope. The frequency of germination was considered for calculating percentage of seed germination. Mean values were obtained from five replicates of each treatment. Experimental design was completely randomized and data were analyzed by analysis of variance and correlation analysis with Statview (Abacus Concept, Inc, Berkeley, CA). The germinated embryos formed PLBs and the resultant PLBs were maintained for another two passages at 4 wk interval on optimum germination condition for further differentiation.

The advance stage PLBs (PLBs with first set of leaflets) and young plantlets were transferred on two different media, namely, MS and Mitra *et al*[12]. The media were supplemented with different organic carbon sources like dextrose, glucose and sucrose (0-4%, w/v) and two different PGRs (NAA and BA) at a concentration of 0-9 µ*M* either alone or in combination. Cultures were maintained at full laboratory illumination. A set of young plantlets was also maintained on medium containing AC (0-0.5%, w/v) along with optimum PGRs to test the effect of AC on culture proliferation and regeneration.

The regenerated plants with 3-4 roots were transferred to ½ MS medium (½ strength of MS mineral salts, full strength of MS organic constituents), sucrose (2%) without PGRs and

Fig. 1—*M. acuminata* plant at a natural habitat in Mokokchung district, Nagaland, India

maintained under normal laboratory condition. The cultures were maintained for 6-7 wk before transferring to potting mix with charcoal pieces, chopped forest litters, and coconut husk and sand (at 1:1 ratio) with a moss topping. The pots were covered with holed transparent poly bags and watered at wk interval for one month and maintained in poly shade with ~ 70% of shading sunlight.

Results and Discussion

In the present study, asymbiotic germination of immature embryos of *M. acuminata* and culture initiation were largely dependent on the developmental stage of immature embryos, illumination at which cultures were maintained, concentrations of sucrose, quality and quantity of PGRs. Seeds at an age of 7-8 WAP exhibited germination of 85% after 135 d (~19 wk) of culture initiation on medium containing sucrose (3%) and formed PLBs (Fig. 2a). Nodular swelling of embryos followed by bursting out from testa was observed after 8 wk of culture. Seeds younger than 6 WAP either did not germinate or exhibited delayed germination; while for seeds aged >9 WAP, germination frequency was comparatively lower (data not presented). The relative time taken after pollination for successful germination of embryos/ovules varied with the species[2-3,11,13]. There has been a critical period of seed development for every orchid species that supported optimum *in vitro* germination. Therefore, it is desirable to determine the right stage to harvest the green pods to achieve an optimal germination. The culture of immature embryos ensures sterility but may require prolong period for germination as the seeds are immature, while the culture of comparatively mature embryos may support better germination but the chances of contamination increases due to establishment of mycorrihizal association[5,14].

The embryos were cultured under two different illuminations. The germination of embryos was better under 50% diffused light (20 µmol m^{-2} s^{-1}) in comparison to full light condition (40 µmol m^{-2} s^{-1}). In general, darkness or low light intensity stimulates the germination of seeds of terrestrial orchids, such as, those of species in the genera *Habenaria*[15]. The seeds of terrestrial orchids germinate *in situ* in the soil in association with a symbiotic relationship with mycorrihizal fungi. Therefore, the preference of seed germination in low light might be beneficial for the efficient establishment of a symbiotic relationship between orchid seed and mycorrihizal fungi in the soil.

Of the different concentrations of sucrose incorporated in the media, concentration of 3% stimulated the optimum germination, while lower and higher concentrations had lower germination (data not presented). Although BA and NAA stimulated the germination of immature embryos of *M. acuminata*, NAA was more effective compared to BA (Table 1). Seed germination of *M. acuminata* on NAA enriched medium was evident after 8 wk of culture and PLBs were formed after 135 d of culture (Table 1). While, seeds maintained on BA rich medium either alone or in combination with NAA delayed germination and germination frequencies were also significantly lower. Li and Xu[16] also reported the stimulatory effect of NAA on the seed germination in conjunction with cytokinin. In *Rhynchostylis gigantean*, NAA (0.2 mg L^{-1}) and

Fig. 2 (a-d)—Different stages of immature seed germination and plant regeneration of *M. acuminate*: a. Asymbiotic germination of immature seeds and formation of protocorm-like bodies (PLBs); b. Regeneration of rooted plants and multiple shoots on regeneration medium; c. Multiple shoot buds and plantlet formation on AC (0.3%) enriched medium; & d. Some potted plants ready to be transferred to the wild.

BA (0.5 mg L^{-1}) supported the optimum germination of seeds. In *Dendrobium* hybrids, Nagaraju *et al*[17] reported the optimal seeds germination followed by development of single leaflet in the PLBs when basal medium was supplemented with BA alone, while BA alone has been found to support better seed germination in *M. khasiana*[3] and *Calanthe tricarinata*[2].

Table 1—Effect of NAA and BA on germination of immature embryos of *M. acuminata**

PGRs conc. (µ*M*)**		Time taken for germination	% germination (±SE)***
NAA	BA	(d)	
0	0	170	10 ± 1.0[g]
2	0	160	75 ± 2.5[b]
4	0	135	85 ± 2.0[a]
6	0	130	62 ± 3.0[c]
8	0	140	62 ± 3.0[c]
10	0	140	50 ± 2.5[e]
0	2	160	71 ± 1.5[b]
0	4	150	66 ± 3.0[c]
0	6	150	57 ± 3.0[d]
0	8	145	55 ± 2.0[d]
0	10	150	50 ± 2.5[e]
2	2	160	40 ± 2.0[f]
2	4	155	40 ± 3.5[f]
6	4	155	62 ± 2.0[c]

Data represent the mean of 5 replicates.

*Immature embryos of 7-8 WAP were cultured on MS medium supplemented with sucrose (3%).

**Only the significant treatments are computed.

***Values followed by the same letter are not significantly different.

The resulted PLBs converted into plantlets within 8-9 wk on regeneration medium and rooted plantlets formed after 12-13 wk of culture. For regeneration of plantlets, three different organic carbon sources dextrose, glucose and sucrose were used. Amongst them, sucrose (3%) supported the healthy plantlet formation where average plant height, number of leaves, number of roots per plant and number of shoot buds formed per subculture recorded to be 2.4 cm, 4.5, 4.0 and 13, respectively (Table 2). However, dextrose and glucose in the entire range did not support the optimum regeneration. Thus, the requirement of the quality and quantity of exogenous supply of the organic carbon sources varied with the species and developmental stages of explants[11].

For regeneration of plantlets and culture proliferation, PLBs and young plantlets were maintained on MS and Mitra *et al* media conjunct with different concentrations of NAA and BA (Table 3). In general, better plantlet regeneration and culture proliferation was achieved on MS medium. Amongst the different concentrations of NAA and BA tested, a combined treatment of NAA and BA at 3 µ*M* each supported healthy regeneration and multiple shoot formation (Table 3 & Fig. 2 b). In single treatment, BA was found to be better compared to NAA; while cultures maintained on NAA alone, supported more root formation but have suppressed multiple shoot and

Table 2—Effect of different organic carbon sources on plant regeneration and culture proliferation of *M. acuminata**

Source of organic carbon (% conc.)	Av. no. of shoots formed per subculture**	Av. plantlet height (cm)**	Av. no. of leaves per plant**	Av. no. of roots per plant**
0	0	0	0	0
Dextrose				
1	6.33±027[c]	1.3±0.20[d]	3.5±0.10[c]	1.5±0.10[f]
2	10.50±0.15[b]	2.0±0.15[b]	4.3±0.15[b]	2.6±0.20[e]
3	3.33±0.10[d]	1.7±0.20[c]	3.3±0.13[c]	2.6±0.10[e]
4	2.50±0.12[d]	1.6±0.10[c]	2.5±0.15[d]	1.5±0.10[f]
Glucose				
1	3.00±0.10[d]	1.4±0.05[d]	3.5±0.10[c]	4.3±0.20[c]
2	6.50±0.20[c]	1.4±0.10[d]	2.5±0.20[d]	4.6±0.15[b]
3	5.66±0.24[c]	1.6±0.15[c]	2.5±0.15[d]	5.5±0.10[a]
4	3.33±0.25[d]	1.4±0.10[d]	2.3±0.10[d]	3.3±0.20[d]
Sucrose				
1	7.50±0.50[c]	1.5 ±0.15[d]	3.5±0.15[c]	3.0±0.15[d]
2	10.00±0.50[b]	1.5±0.10[d]	3.3±0.20[c]	4.3±0.25[c]
3	13.00±1.00[a]	2.4±0.10[a]	4.5±0.20[a]	4.0±0.20[b]
4	6.33±1.00[c]	2.0±0.10[b]	4.0±0.20[b]	2.5±0.10[e]

*On MS medium containing NAA+BA (3 µ*M* each) and AC (0.3%).

Data represents the mean of 5 replicates and data collected after 70 d of culture on the medium.

**Standard error; values followed by the same letter are not significantly different.

Table 3—Effect of basal media and PGRs on plant regeneration and culture proliferation of *M. acuminata**

PGRs conc. (μ*M*)		No. of shoots#		Plant height# (cm)		No. of leaves#		No. of roots#	
NAA	BA	M	Mi	M	Mi	M	Mi	M	Mi
0	0	-	-	-	-	-	-	-	-
0	3	3.2±0.4^{d}	2.0±0.3^{e}	2.1±0.3^{b}	1.3±0.2^{b}	3.2±0.3^{c}	3.3±0.2^{a}	1.0±0.1^{d}	1.0±0.1^{c}
0	6	4.3±0.3^{d}	8.1±1.0^{b}	1.5±0.2^{d}	1.5±0.1^{a}	2.0±0.2^{d}	2.0±0.1^{c}	1.0±0.2^{d}	1.0±0.2^{c}
0	9	8.5±0.5^{c}	6.3±0.5^{c}	1.6±0.3^{c}	1.4±0.1^{b}	3.1±0.3^{c}	2.0±0.1^{c}	1.0±0.1^{d}	2.0±0.2^{b}
3	0	2.0±0.3^{c}	10.5±1.5^{a}	2.4±0.2^{b}	1.2±0.2^{b}	3.2±0.1^{c}	2.3±0.3^{c}	1.0±0.1^{d}	2.0±0.1^{b}
6	0	3.1±0.4^{d}	8.3±1.0^{b}	3.1±0.3^{a}	1.1±0.2^{c}	3.2±0.2^{c}	2.1±0.2^{c}	4.0±0.1^{b}	3.0±0.3^{a}
9	0	2.0±0.2^{e}	6.1±1.1^{c}	1.1±0.2^{d}	1.0±0.1^{c}	2.0±0.1^{d}	2.0±0.2^{c}	1.0±0.2^{d}	1.0±0.1^{c}
3	3	18.0±1.0^{a}	3.0±0.5^{d}	2.4±0.1^{b}	1.5±0.3^{a}	4.5±0.2^{a}	3.1±0.3^{a}	4.0±0.2^{b}	2.0±0.2^{b}
3	6	6.1±1.0^{c}	7.1±0.3^{b}	1.9±0.4^{c}	1.6±0.3^{a}	3.0±0.2^{c}	3.0±0.3^{b}	4.0±0.3^{b}	2.0±0.1^{b}
3	9	3.1±0.5^{d}	5.5±0.5^{c}	1.8±0.2^{c}	0.8±0.2^{c}	2.1±0.1^{d}	3.2±0.4^{a}	1.0±0.1^{d}	1.0±0.1^{c}
6	3	9.2±1.5^{b}	5.5±0.0^{c}	2.9±0.3^{a}	1.5±0.1^{a}	4.2±0.2^{b}	2.1±0.3^{c}	6.0±0.4^{a}	2.0±0.1^{b}
6	6	10.1±1.5^{b}	6.3±0.5^{c}	1.6±0.1^{c}	1.2±0.1^{b}	3.1±0.3^{c}	2.1±0.2^{c}	2.0±0.2^{c}	1.0±0.1^{c}
6	9	8.5±1.0^{c}	5.3±0.3^{c}	1.9±0.1^{c}	1.2±0.1^{b}	3.1±0.3^{c}	2.1±0.3^{c}	2.0±0.2^{c}	1.0±0.1^{c}
9	3	10.3±1.0^{b}	6.5±0.4^{c}	1.7±0.2^{c}	1.4±0.2^{b}	3.1±0.2^{c}	2.1±0.1^{c}	1.0±0.1^{d}	1.0±0.1^{c}
9	6	3.0±0.5^{d}	2.0±0.2^{e}	2.1±0.3^{b}	1.5±0.3^{a}	3.1±0.2^{c}	2.0±0.2^{c}	0.0	0.0
9	9	8.3±0.5^{c}	4.1±0.3^{d}	1.8±0.3^{c}	1.3±0.2^{b}	2.1±0.2^{d}	2.0±0.1^{c}	0.0	0.0

*Media containing sucrose (3%) and AC (0.3%).
#M: On MS medium; Mi: On Mitra *et al* medium.
Data represents the mean of 5 replicates and data collected after 70 d of culture on the medium.
Values followed by the same letter are not significantly different.

leaf formation. Cultures on BA enriched media although have moderately high number of shoots but did not support the induction of healthy root formation, whereas healthy shoot formation with multiple roots was observed in the presence of both NAA and BA in the medium. Several workers supported the view that change in culture conditions and media could alter the pattern of organogenesis in orchids and such behaviour could be judiciously exploited to achieve desirable response in orchid taxa by altering the nutrient regime[5,11,18].

Besides above factors, AC at different concentrations was incorporated (0-0.5%) in the regeneration medium. In general, cultures maintained on medium enriched with lower concentrations of AC (0.1 and 0.2%, w/v) did not support healthy culture growth and culture proliferation and performances were found to be poorer when compared with the control medium (without AC). But higher concentration (0.3%) of AC facilitated the culture proliferation, induction of newer shoot buds and enlargement of pseudobulbs. In comparison to 13 shoot buds/PLBs formed (Fig. 2 b) under control medium (without AC), about 18 shoot buds/PLBs (Fig. 2 c) were observed onto AC (0.3%) enriched medium. The optimum response was achieved on MS medium enriched with sucrose (3%), NAA and BA (3 μ*M* each in combination) and AC (0.3%) (Fig. 3),

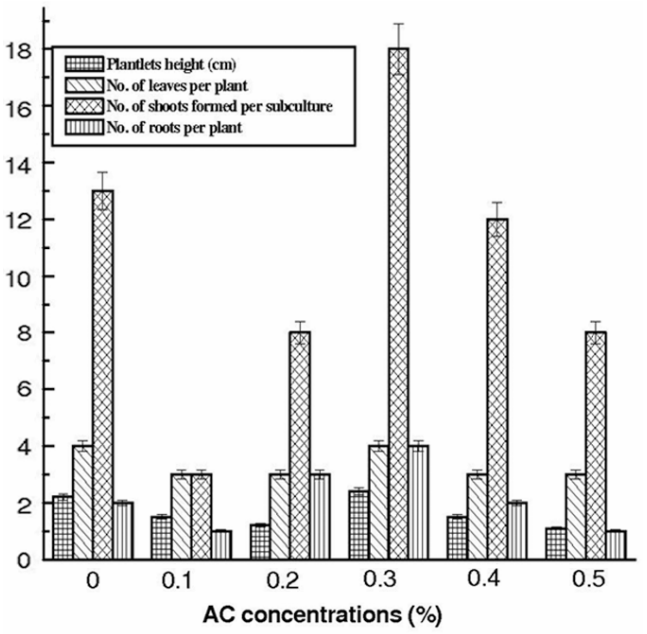

Fig. 3—Effect of activated charcoal on plant regeneration and culture proliferation of *M. acuminata*.

where an average 4.5 leaves, 4 roots per plants and 18 shoot buds formed per subculture. The synergistic effect of cytokinins and auxin in plant formation and culture proliferation, as observed in the present study, has also been reported in *M. khasiana*[3] and *Micropera pallida*[19]. However, in Japanese *Calanthe* species,

use of auxin and cytokinin alone favoured shoot formation[20]. The positive effect of AC in shoot induction has also been documented in *M. khasiana*[3], and *Coelogyne viscosa*[21].

The regenerated plants with 3-4 roots were transferred to ½ MS medium with sucrose (2%) without PGRs and maintained under normal laboratory condition. The cultures were maintained for 6-7 wk before transferring to potting mix with charcoal pieces, chopped forest litters, and coconut husk and sand (at 1:1 ratio) with a moss topping (Fig. 2 d). The pots were covered with holed transparent poly bags and watered at wk interval for one month and maintained in polyshade with ~70% of shading sunlight for 2 months before transferring to the wild. About 75% transplants survived in the wild after 2 months of transfer.

The protocol established in the present study was efficient and repeatable, and would help to propagate this therapeutically important orchid in order to keep pace with the need of time and also to keep off the species from the threat of extinction.

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