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In vitro propagation of threatened terrestrial orchid, *Malaxis khasiana* Soland ex. Swartz through immature seed culture

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Rapid *in vitro* propagation of the terrestrial orchid, *M. khasiana* through immature seed culture was achieved. Immature seeds of 8-9 week after pollination (WAP) cultured on MS medium (2% sucrose) supplemented with 500 mgl^{-1} casein-hydrolysate and 1 μ*M* N^6-benzyladenine (BA) exhibited germination of 75% seeds after 107 days of culture and subsequently supported the development of PLBs. Subsequent culture on MS medium enriched with 6 μ*M* of indole-3-acetic acid (IAA), 18 μ*M* each of BA and kinetin induced multiple shoots and plantlets. Transfer of PLBs to MS medium with 0.1% activated charcoal (AC) facilitated rapid proliferation of PLBs, while AC at 0.2% favored shoot bud induction and rhizome enlargement. The plantlets, developed on medium with IAA, BA and kinetin, after hardening *in vitro* for 8-10 weeks were planted in community pots and transferred to poly-house. The plantlets showed 65% survival under field conditions.

Keywords: Activated charcoal, Immature seed culture, *In vitro* hardening, Mass multiplication, Plant growth regulators

Orchids, besides their ornamental prices, are therapeutically important as sources of curare compounds to several ailments. The propagation of orchids is hampered because of low viability of seeds and mandatory mycorrhizal association for seed germination. The orchid genus *Malaxis* comprising about 300 species have distribution throughout the tropical to temperate climate regions. Of the 19 species of the genus represented in India, most are components of Ayurvedic drug preparations. The dried pseudobulbs are important ingredient of Ashtavarga drugs used in the preparation of Chyvanprash, an energetic herbal tonic[1] and to cure tubercolosis[2].

Malaxis khasiana Soland ex. Swartz is a threatened terrestrial orchid inhabiting the forest floor having distribution in North Eastern parts of India. The loss of forest cover due to anthropogenic invasions and animal grazing, and extensive collection of the rhizome for drug preparations turned the species to the endangered category. The conventional propagation of the species is too slow and unable to overcome the threat of extinction. *In vitro* propagation strategies are a viable alternative for the rapid propagation of plants at threat. Rapid *in vitro* propagation of several endangered orchids has been reported[1, 3, 4]. The present study describes an efficient *in vitro* propagation and mass multiplication protocol for the threatened species, *M. khasiana*.

Materials and Methods

The immature seeds from green pods of different developmental stages at one week interval starting from four week after pollination (WAP) to 14 WAP were collected from poly-house grown plants. The pods were sterilized by dipping in 0.05% (w/v) aqueous solution of $HgCl_2$ for 5 min followed by 4-5 washes with sterile water. Thereafter, the immature seeds were scooped out under aseptic conditions from the sterilized green pods and cultured on different basal media fortified with different levels of various supplements. Two basal media viz. Murashige and Skoog[5] (MS) medium and Mitra *et al*. medium[6] were used for the present investigation. These media were supplemented with (w/v) (0-3%) sucrose, 0-1000 mgl^{-1} (w/v) casein-hydrolysate (CH) and different level of plant growth regulators (PGRs) viz. 0-3 μ*M* α-naphthalene acetic acid (NAA) and N^6-benzyladenine (BA) either singly or in combination. The *p*H of the media was adjusted to 5.6 using 0.1 N NaOH and HCl gelled using 0.7% agar (make: Hi-media) before autoclaving at 1.05 kg cm^{-2} and 121° C for 20 min. Cultures were maintained at 24°±1° C and

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40 μmol m^{-2} s^{-1} light intensity (12:12 hr L:D) provided with white cool florescent tube light. For each treatment 25 numbers of culture vials were maintained and the cultures were subcultured at four week interval unless otherwise mentioned.

The protocorm-like bodies (PLBs) developed from the cultured immature seeds maintained on germination medium until emergence of first leaflets. Those PLBs were transferred on MS medium containing (w/v) 3% sucrose, (500 mgl^{-1}) CH and different levels (0-27 μM) of indole-3-acetic acid (IAA), NAA, BA and kinetin (Kn) either singly or in combination for regeneration of plantlets and mass multiplication (Table 3). Alternatively, the young plantlets and PLBs were cultured on MS media containing (w/v) 0-0.3% activated charcoal (AC) in conjunction with optimum levels of PGRs to study the effect of AC on regeneration and mass multiplication. The plantlets (> 5 cm height) (with 3-5 roots) were hardened *in vitro* by transferring cultures in vials containing $1/10^{th}$ MS liquid media and sterile charcoal pieces, chopped coconut husk and chopped litters (1:1:1 ratio), and maintained for 8-10 week. The *in vitro* hardened plants are transferred to potting mix containing charcoal pieces, coconut husk and sterilized forest litter (1:1:1 ratio) and maintained in poly-house for 6-8 week before transferring to field. All the experiments were repeated thrice and identical physical conditions were maintained for the entire period of investigation and for every replicate.

Results and Discussion

In the present studies on *M. khasiana,* non-symbiotic seed germination and culture initiation largely depended on the culture media, PGRs and age of the immature seeds. MS medium was superior to Mitra *et al.* medium (Table 1). Seeds at an age of 8-9 WAP exhibited germination of 75% seeds after 107 days of culture on MS medium with 500 mgl^{-1} CH, 2% sucrose and 1 μ*M* BA. A nodular swelling of the seeds after 60 days of culture was the first sign of seed germination. Seeds over 10 WAP in most of the cases failed to germinate and in some cases only swelling observed after a prolonged period of culture. While, seeds sown at or below 7 WAP did not germinate, however in some cases swelling observed but without PLBs formation. The relative time taken after pollination wherever embryos/ovules could be successfully germinated seems to be varying with species[3,7,8]. Jamir *et al.*[4] achieved successful germination by culturing *Cymbidium iridioides* seeds of 120 days old while Temjensangba and Deb[8] could raise the culture from 16 WAP of *Cleisostoma racemiferum*.

Of the different growth regulators, medium with 1 μ*M* BA was most suitable for germination and was followed by medium having 3 μ*M* BA and 1 μ*M* NAA with 66% germination and PLBs formation (Table 2). Medium with 1 μ*M* BA rich medium supported healthy germination and PLBs formation (Fig. 1). The PLBs started converted into plantlets

Table 1— Effect of seed age and medium* on immature seed culture of *M. khasiana*
[Values are mean of three replicates of 25 cultures of each treatment]

Seed age (WAP)	Germination time (days)		Germination (%) (±SE)		Type of response
	MS	Mitra *et al*[6]	MS medium	Mitra *et al*[6] medium	
4	-	-	-	-	No response
5	-	-	-	-	No response
6	-	-	-	-	No response
7	140	145	30 ±2.0	20 ±2.0	Nodular swelling but degenerated subsequently
8	107	115	75 ±2.5	35 ±3.0	Nodular swelling followed by PLBs formation
9	107	120	74 ±2.0	27 ±2.0	As above
10	110	120	60 ±3.0	20 ±2.0	As above but PLBs failed to form plantlets
11	120	125	40 ±3.0	15 ±2.5	Deformed PLBs formation
12	140	140	20 ±3.0	15 ±2.0	Culture degenerated subsequently
13	-	-	-	-	No response
14	-	-	-	-	No response

* Media with 2% sucrose, 500 mgl^{-1} and 1 μ*M* BA
Data collection started after 60 days of culture and one week interval

within 7-8 week on regeneration medium and after 10-12 week of culture rooted plantlet formed. The regeneration of plantlets and PLBs proliferation was better on medium with a combination of IAA, BA and Kn rather than the use of BA alone for germination (Figs 2 and 3). Culture of the PLBs on MS medium with 6 μM IAA, 18 μ*M* each of BA and Kn yielded as many as 18 PLBs/shoot buds per subculture (Table 3). Singly presence of BA in the regeneration medium supported shoot bud formation but, singly treatment of Kn triggered simultaneous shoot bud and secondary PLBs formation. While, incorporation of NAA either alone or in combination with other growth regulators did not support plant regeneration. Addition of AC into the multiplication medium influenced proliferation of PLBs and induction of shoot buds. Medium with 0.1% AC favored proliferation of PLBs, while, 0.2% supported shoot bud induction and rhizome enlargement. But the higher concentration of AC (0.3%) cultures turned brown and degenerated subsequently.

Table 2— Effect of plant growth regulators* on culture of immature seeds (8-9 WAP) of *M. khasiana*

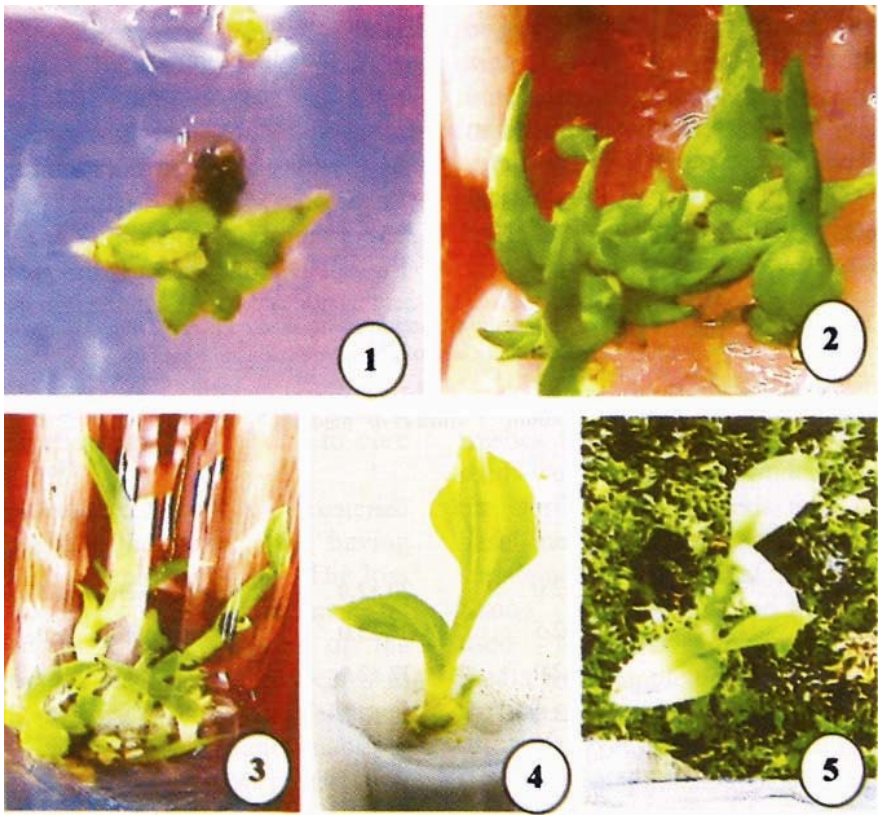
[Values are mean of three replicates of 25 cultures of each treatment]

PGRs (μM)		Germination time (days)	Germination (%)(±SE)	Emergence of 1st leaf (days)
NAA	BA			
0	0	-	-	-
0	1	107	75 ±2.5	135
0	2	110	40 ±3.0	145
1	2	115	25 ±2.0	140
1	3	103	66 ±2.5	138
2	1	106	60 ±3.0	135
2	2	110	45 ±2.0	140
2	3	115	40 ±2.0	145
3	3	115	20 ±3.0	-

Data computed only from the responding concentrations/combinations

* MS medium with 2% sucrose, 500 mgl^{-1} CH

Data collection started after 60 days of culture and one week interval



Figs.1-5— Immature seed culture of *M. khasiana* (1)— Immature seed culture of *Malaxis khasiana*. 1. PLBs development from immature seeds on MS medium with 2% sucrose, 500 mgl^{-1} CH and 1 μ*M* BA; (2)—.Proliferation of PLBs and shoot buds on MS medium with 2% sucrose, 500 mgl^{-1} CH and 1 μ*M* BA; (3) — Multiple shoots formation on MS regeneration medium with 3% sucrose, 6 μ*M* IAA, 18 μ*M* each of BA and Kn, (4) — Well differentiated plantlet on regeneration medium ready for hardening and (5) — Plant in community pot.

Table 3— Effect of PGRs on *in vitro* regeneration of plantlets of *M. khasiana**

[Values are mean of three replicates of 25 cultures of each treatment]

PGRs (μM)				No. of shoot buds formed/PLBs	Type of response
IAA	NAA	BA	Kn		
0	0	0	0	-	No regeneration
6	-	-	-	3	Shoot bud formation
-	27	-	-	3	Shoot bud formation
-	-	9	-	1	Single shoot bud formation
-	-	18	-	2	Shoot bud formation
-	-	27	-	5	As above
-	-	-	9	3	Shoot bud formation
-	-	-	18	5	Simultaneous shoot buds and PLBs formation
-	-	-	27	4	PLBs mediated regeneration
6	-	9	-	5	Direct shoot bud induction
6	-	18	-	4	As above
6	-	27	-	1	Single PLBs formation
6	-	-	9	1	Single shoot bud induction
6	-	-	18	4	As above
6	-	-	27	4	Multiple shoot bud formation
6	-	9	9	11	Multiple shoot bud and PLBs formation
6	-	9	18	5	Direct shoot bud formation
6	-	18	9	6	As above
6	-	18	18	18	Simultaneous multiple shoot bud and PLBs formation
6	-	18	27	5	Direct shoot bud induction
6	-	27	9	7	Simultaneous shoot bud and PLBs formation
6	-	6	18	2	Shoot bud induction
6	-	27	27	2	Direct PLBs formation

* MS medium with 3% sucrose

Data computed only from the responding concentrations/ combinations

Data collected after 7-8 week of culture on regeneration medium

Effectiveness of BA in the germination of immature seeds as the present study has been documented in orchid's viz. *Cleisostoma racemiferum*[8]. However, the potential of different growth regulators in seed germination has been reported in *Vanda coerulea*[9]. In contrast, inhibition of germination by combination of NAA, BA and Kn has been reported in *Aerides rosea*[10]. The synergistic effect of cytokinins and auxin in PLBs proliferation and plant formation as in the present study has been reported in another species, *Malaxis acuminata*[1]. However, the influence of cytokinin alone in PLBs formation has also been reported in *Vanda*[11]. In Japanese *Calanthe* species use auxin and cytokinin singly favored shoot formation[12]. As in the present study, positive modification of AC in shoot induction has been documented in *Coelogyne viscose*[13]. Nevertheless, AC was inhibitory in *Malaxis acuminata*[1].

The plantlets with 3-5 roots (>5 cm) developed on regeneration medium (Fig. 4) were hardened *in vitro* for 8-9 week as described in materials and method. During *in vitro* hardening, the roots were attached to the substratum in the culture vessels. The hardened plants after transfer to community pots were maintained in the polyhouse for 6-8 weeks and subsequently transferred to wild (Fig. 5). The transplanted regenerates showed 65% survival.

The established protocol is efficient to propagate this therapeutically important orchid which is necessary to keep pace with the need and also to keep off the species from extinction.

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