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STUDIES ON *In vitro* MORPHOGENETIC POTENTIAL OF NODAL SEGMENTS OF *Malaxis acuminata* D. Don

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ABSTRACT

***Malaxis acuminata* is a therapeutically important terrestrial orchid. Due to various anthropogenic activities and over-collection for medicinal purposes the species is under threat in its natural habitat. A successful attempt was made to propagate this species using nodal segments from axenic source as alternative explants. Invocation of meristematic loci from the cultured nodal segments initiated within three weeks of culture on MS medium fortified with sucrose (3%, w/v), NAA and BA (3 µM each in combination). Under this condition as many as 18 shoot buds developed. The resultant shoot buds converted into plantlets with well developed roots on MS medium supplemented with sucrose (3%), NAA and BA (3 µM each in combination) where average plant height and number of leaves, shoots and roots were 2.4 cm, 4.5, 18.0 and 4.0, respectively. The hardened regenerates were transferred to community potting mix prepared by mixing charcoal pieces, chopped forest litter, coconut husk, sand and black soil in equal proportion. About 75% of transplants survived after 2 months of transfer.**

Keywords: *Malaxis acuminata*, medicinal orchid, micro-propagation, nodal explants

INTRODUCTION

Many threatened plant taxa have been propagated successfully through tissue culture and reintroduced into the wild thereby ameliorating their status in nature. Plant tissue culture is a useful technique visualized for the conservation of rare/threatened/endangered species, in mass multiplication of the species with reproductive problems and/or having unsustainable population in natural habitat. Different explant sources like seeds, foliar explants, rhizomes, roots, inflorescence, etc. have been used for *in vitro* propagation of various plant species (Seeni and Latha, 1992; Deb and Sungkumlong, 2010; Pongener and Deb, 2011a; Deb and Pongener, 2012). A wide range of economically important, threatened and endangered plants have now successfully been propagated using *in vitro* techniques like micropropagation and somatic embryogenesis (Temjensangba and Deb, 2006; Deb and Temjensangba, 2006a,b, 2007a; Yarra *et al.*, 2010; Yapo *et al.*, 2011). *In vitro* propagation and conservation of plant germplasm is increasingly being considered as a more practical option compared to conventional approaches.

Malaxis acuminata D. Don is a small medium-sized terrestrial orchid up to 30 cm high, with pseudo-bulbs at the base. It grows in forest litter in shady areas and usually flowers during the month of June-July. Of the 19 species of the genus existing in India, some, including *Malaxis acuminata*, are used in 'Ayurvedic' drug preparation. Dried pseudobulbs are important ingredient of 'Ashtavarga drug' used in the preparation of herbal tonic 'Chyavanprash', and to cure tuberculosis. The species grows on

decayed forest litter of primary forest. Loss of forest cover due to anthropogenic activities, animal grazing and extensive collection of psuedobulb for drug preparation has reduced the species to endangered status. Present communiqué describes a successful attempt to propagate *Malaxis acuminata* from nodal segments.

MATERIALS AND METHODS

Explant source

Nodal explants were collected from *in vitro*-raised etiolated plants. Stock cultures were raised by culturing 7-8 weeks old immature embryos on MS medium (Murashige and Skoog, 1962) supplemented with sucrose (3%, w/v), 4 µM NAA. Germinated embryos formed protocorm-like bodies (PLBs) after 135 days of culture which latter developed into plantlets on MS medium fortified with sucrose (3%), NAA and BA (3 µM each in combination). Plantlets were taken out in the laminar flow cabinet, the leaves/scales removed and cut into one or two node segments to be used as explants to initiate the culture. The processed nodal segments were soaked in sterilized distilled water till cultured on initiation medium.

Initiation of culture

Processed nodal segments were cultured on agar congealed (0.8%) MS medium with sucrose (3%) and NAA and BA (0-9 µM) either singly or in combination. Twenty explants were maintained per treatment replicated thrice in a completely randomized design. Cultures were maintained at 25±2°C, 40 µmol m^{-2} s^{-1} illumination and 12:12 h light: dark regime. Sub-culturing was done at 4-5 week interval unless mentioned otherwise. *In vitro* response was evaluated based on the percentage of explants responding and number of propagules formed in the culture after specific period of time.

Regeneration of plants and culture proliferation

The PLBs and shoot buds formed from the cultured nodal segments were maintained for another two passages for further differentiation and proliferation on optimum growth conditions. The advanced stage PLBs/ shoot buds developed from the cultured nodal explants were transferred on regeneration medium for regeneration of plantlets and culture proliferation. The advanced stage PLBs/shoot buds developed from nodal explants were maintained on medium for plant regeneration and culture proliferation.

Young plantlets/shoot buds were transferred initially on two different media namely MS and Mitra *et al.* (Mitra *et al.*, 1976) media both supplemented with sucrose 3%, and NAA and BA ranging from a concentration of 0-9 µM either singly or in combination. Subsequently PLBs were transferred on different strengths of MS salt medium with different supplements and different quality and quantity of PGRs for plant regeneration and culture proliferation.

Hardening and transplantation of regenerates

The regenerated plantlets were maintained on optimum regeneration condition for 2-3 passages for culture proliferation and plantlet growth. About 3 cm long plantlets with distinct pseudobulb, 4-5 leaves and 4-5 roots from regeneration medium were taken out from the regeneration medium and subjected to *in vitro* hardening. The well rooted plantlets were taken out from the regeneration medium and transferred on 1/2 strength MS medium containing sucrose (0-2%, w/v) and devoid of any PGRs and maintained in normal laboratory conditions (40 µmol m^{-2} s^{-1} illumination and 25±2°C) for 6-7 week prior to transferring to the potting mix. The hardened plantlets were then transplanted onto community potting mix (CPM) of charcoal pieces, chopped forest litter, coconut husk, sand and black soil in equal ratio with a moss topping.

The CPM were covered with perforated transparent polybags and watered at weekly interval for one month. Plants were fed with $1/10^{th}$ MS salt solution once in a week for 3-4 weeks and maintained under 70% polyshade with, before transferring them to the wild.

RESULTS AND DISCUSSION

Initiation of cultures

Sprouting of shoot buds/PLBs from nodal region of segments initiated within 3 weeks of culture (Plate 1a,b). Medium enriched with NAA supported fewer shoot buds/PLBs formation while BA enriched medium gave moderate response (Table 1). Combined treatments were more effective than individual treatments. A mean of 18 shoot buds/PLBs was recorded at a single node on MS medium supplemented with sucrose (3%), NAA and BA (3 µM each) (Plate 1c). The findings are in agreement with Kosir *et al.* (2004) who in case of *Phalaenopsis* reported that combined treatment of BA (2 mg L^{-1}) and NAA (0.5 mg L^{-1}) were optimum requirement for breaking the axillary buds and formation of multiple shoot buds. Further, they observed that incorporation of NAA was promotory. Tisserat and Jones (1999) and Roy and Banerjee (2003) observed that an appropriate combination of NAA and BA stimulated multiple shoot bud formation. However, Arditti and Ernst (1993) and Pongener and Deb (2011a) reported that BA promotes morphogenetic response, whereas the addition of NAA reduces induction and regeneration. In present study about 90% explants responded positively with the sprouting of shoot buds/PLBs from the nodal regions under optimum conditions (data not shown). The PLBs and shoot buds formed from cultured nodal segments were maintained for another two passages for further differentiation and proliferation on optimum initiation conditions. The advanced stage PLBs, shoot buds and tiny plantlets, developed on initiation medium, were transferred on regeneration medium for plantlets formation and culture proliferation. The advanced stage PLBs/shoot buds, developed from nodal explants, were maintained on medium for plant regeneration and culture proliferation.

Plantlet regeneration and culture proliferation

Effect of basal media: The differentiated PLBs/shoot buds and young plantlets were maintained on two different media viz., Mitra *et al.* and MS media with different growth adjuncts. In preliminary study it was observed that MS medium supported better regeneration and multiplication of culture compared to the Mitra *et al.* medium. The cultures maintained on MS medium differentiated faster and gave higher

Table 1: Effects of PGRs on morphogenetic response of axenic nodal explants of *M. acuminate* D. Don

Concentration of PGRs (µM)		Time for initial response (days)	No. of shoots formed (±SE)*	No. of roots formed (±SE)*	Type of response**
NAA	BA				
0	3	30.3	8.3^{c} (1.0)	0.0	Swelling of explants, shoots formed in cluster from node
0	6	25.5	5.3^{d} (0.5)	2.3^{b} (0.2)	Shoot buds formed from node, a few converted to rooted plantlets.
0	9	35.3	16.6^{b} (0.5)	0.0	Shoot buds light green, many converted to plantlets
3	0	28.6	4.3^{d} (1.0)	5.0^{a} (0.3)	Plantlets with healthy roots/shoots etiolated; leaves small
6	0	18.3	5.0^{d} (0.5)	4.6^{a} (0.2)	Shoot bud formed from node, a few converted to plantlets; leaves small; plantlet thin and elongated.
3	3	21.6	18.3^{a} (1.5)	3.3^{b}(0.3)	Shoot bud formed in clusters, many converted to tiny plantlets.
3	6	32.3	8.3^{c} (1.0)	1.0^{c}(0.2)	Shoot buds formed in cluster and all converted to plantlet
6	3	18.6	4.3^{d} (0.5)	2.3^{b} (0.2)	Shoots small, formed from node, unhealthy
6	6	49.3	10.5^{c} (1.5)	2.3^{b} (0.2)	Shoots/PLBs developed from node.
6	9	30.5	8.3^{c} (1.0)	0.0	Shoots formed from node in cluster. Few converted to plantlet with small leaves.
9	6	40.5	5.3^{d} (1.0	0.0	Shoots formed at the node. Few shoots converted to healthy plantlets.

*Standard error; Values followed by the same *letters* are not significantly different from each other.

**On MS medium with 3% sucrose;

Data represents the mean of three replicates. Only the significant treatments are computed.

number of secondary shoots/PLBs (Table 2). Subsequent experiments were conducted to study the effects of different strengths of MS medium on plant regeneration and growth. At lower strengths ($1/4^{th}$, 1/2 strengths) plant growth was stunted and accompanied by fewer new shoots and bud formation. Maximum plant height (~2.4 cm) and shoot bud formation (18 shoot buds plant^{-1} subculture^{-1}) was achieved on full MS medium (Table 3, Plate 1d). This differential growth pattern probably could be attributed to the difference in chemical constituents in the MS medium. Chen *et al.* (2004), Temjensangba and Deb (2005) and Pongener and Deb (2009, 2011b) have argued that the change in culture conditions and media could alter the pattern of organogenesis in orchids and such behaviour can be judiciously exploited to achieve desirable response in many orchid taxa by altering their nutrient regime.

Effect of PGRs: The quality and quantity of PGRs exhibited pronounced effect and elicited different responses in seedling development. Inclusion of PGRs in regeneration medium was obligatory for the successful regeneration of plantlets and mass multiplication in the present study. In absence of PGRs, no regeneration and cultures degeneration occurred subsequently. Single treatments of different PGRs at lower concentrations resulted in stunted growth of plantlets while at higher concentrations regenerants showed etiolated growth. None of the single concentration treatment could support the formation of multiple shoots and only combined treatments of different PGRs produced multiple shoots/ propagules. Amongst the different quality and quantity of PGRs used, NAA and BA (3 + 3 µM in combination) supported optimum regeneration and mass multiplication of plantlets which produced multiple shoots buds. As many as 18 shoots/shoot buds developed per explants per subculture (Table 2). Under this condition, the average plant height and the number of leaves, shoots and roots were 2.4 cm, 4.5, 18.0 and 4.0, respectively, on MS medium against 1.5 cm, 3.1, 3.0 and 2.0, respectively, on Mitra *et al.* medium under otherwise identical conditions.

Chen *et al.* (2004), Temjensangba and Deb (2005) and Pongener and Deb (2011b) have argued that the change in culture conditions and media could alter the pattern of organogenesis in orchids. Vij

Table 2: Effect of basal media and PGRs on plant regeneration and culture proliferation of *M. acuminate* D. Don*

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

*Media containing sucrose (3%); [#]M: On MS medium, Mi: On Mitra *et al.* medium.
Data represents the mean of three replicates and data collected after 70 day of culture on the medium.
Values super-suffixed by same *letters* are not significantly different from each other.

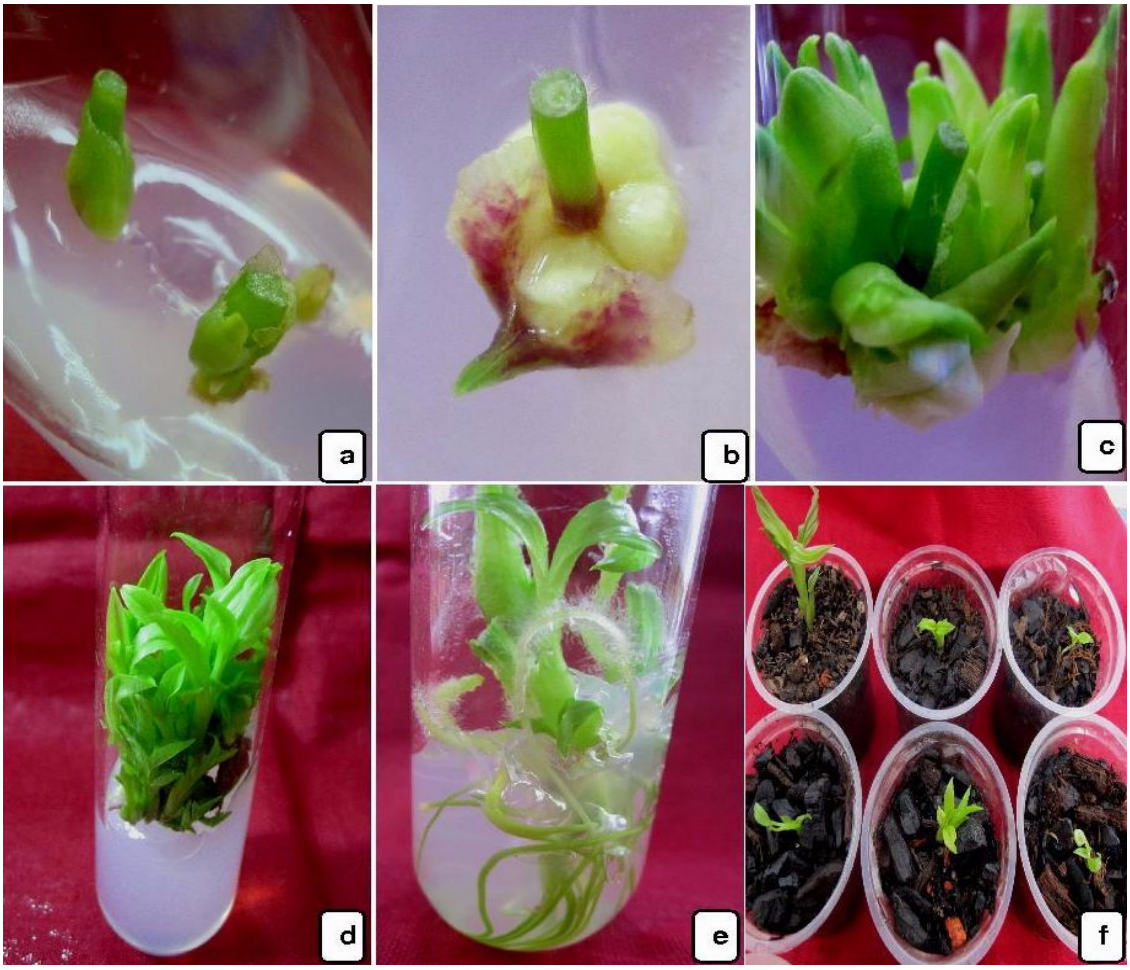

Plate 1: Different stages of *in vitro* propagation of *Malaxis acuminata* using nodal segments as explants source. a) Processed nodal segments cultures on initiation medium, b) Invocation of merismetic loci/shoot buds from the cultured nodal segments, c) Formation of multiple shoot buds on initiation medium, d) Multiple plantlets formation on regeneration medium, e) Rooted plants under hardening condition, and f) Regenerated plants in community potting mix.

Table 3: Effect of basal medium strength on plant regeneration and culture proliferation of *M. acuminate* D. Don.

Strength of MS medium	Plantlet height (cm)*	No. of leaves*	No. of shoots*	No. of roots*	Type of response**
0	0	0	0	0	Culture degenerated
1/4th	1.4(0.2)	4.0(0.2)	1.0(0.1)	2.0(0.3)	Leaves small, dark green, pigmentation developed at the base.
1/2	1.8(0.15)	4.0(0.3)	5.0(0.2)	4.0(0.2) .	Leaves small, dark green, leaf blade fully opened, pigmentation developed at the base, roots with healthy root hairs
3/4th	2.1(0.15)	5.0(0.3)	9.0(0.5)	3.0(0.2)	Leaves small, dark green, leaf blade opened fully, plantlets healthy.
Full	2.4(0.1)	4.5.0(0.2)	18.0(1.0)	4.0(0.2)	Healthy plantlets with many shoot buds, violet pigmentations formed, pseudo-bulb swollen.

*±Standard error; **On MS medium containing sucrose (3%) and NAA+BA (3 µM each in combination)
Data harvested after 40 days of culture; Data represents the mean of 3 replicates.

and Aggarwal (2003) reported that NAA favoured the development of multiple shoots/PLBs in *Vanda coerulea*. Bhadra and Hossain (2004) reported highest number of multiple shoot buds formation from nodal segment of *Micropera pallida* when medium was supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} BA. PGRs like BAP, singly or in combination with IAA, have been reported to be best used for initiation of cultures and development of healthy plantlets from leaf explants of *Saccolabium papillosum* (Kaur and Vij, 2000).

Hardening and field trial of the regenerates

The hardening of *in vitro*-raised plantlets is essential for better survival and successful establishment. In other words, the survival is determined by the hardening of plantlets. Loss of micro-propagated plants after their transfer to field are attributed to low humidity, high light intensity and non-sterile condition of *in vivo* environment (Paul, 1999; Deb and Imchen, 2010, Lavanya *et al.*, 2009). Conventionally, the tissue-raised plants are hardened by transferring on a low nutrient medium having low organic carbon sources and maintained at high light intensity.

In present study, well developed plants with characteristic pigmentation of *Malaxis acuminata* (Plate 1e) were transferred on 1/2 strength MS medium containing sucrose (2%) but free from any PGRs and maintained for ~6-7 weeks under normal laboratory conditions. The plants hardened without sucrose were very short and tended to degenerate, but when sucrose was added at lower concentration the health conditions improved and the plants showed better survival in potting mix. The hardened plants were then transferred to CPM. To transplant the hardened plantlets, CPM was prepared by mixing different substrates like charcoal pieces, chopped forest litter, coconut husk, sand and black soil (in 1:1 ratio) with a moss topping. After transferring the hardened plants to CPM they were covered with holed transparent polybags. The potted plants were maintained in polyhouse (ca. 70% filtered light) and fed with MS liquid salt solution ($1/10^{th}$ strength) weekly for 2-3 weeks (Plate 1f). The potted plants were left in normal full day light for about 7-8 weeks before transfer to the wild. During this process plantlets turned deep green and developed pigments. About ~75% transplants survived and formed fully developed plants 2 months after potting. Feeding plantlets with nutrient salt solution is reported to be beneficial for orchid survival and growth (Kumaria and Tandon, 1994; Deb and Temjensangba, 2005, 2006b, 2007b; Temjensangba and Deb, 2005; Pongener and Deb, 2009, 2011a,b). The acclimatized plants were transferred to the Departmental Botanical Garden and their performances monitored at regular intervals.

In the present investigation, the plantlets in hardening condition developed new roots. These newly developed roots got attached to the support medium with time, and vigorous plantlet growth was observed. The roots attached themselves mostly to the charcoal pieces and moss, which strongly suggest the suitability of material for the purpose. It was observed that the transplanted-regenerates were dark green and healthy with emergence of new roots and leaves just after one month of transfer in the potting mix.

Conclusions: During the present investigation, protocol has been established for culture initiation from nodal explants from *in vitro* sources of *Malaxis acuminata*, regeneration of plantlets and mass multiplication. This technique opens up an alternative route for *in vitro* mass multiplication of this economically important orchid species.

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