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ORIGINAL PAPER

Adventitious shoot induction from cultured internodal explants of *Malaxis acuminata* D. Don, a valuable terrestrial medicinal orchid

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Abstract *Malaxis acuminata* is a terrestrial orchid that grows in shady areas of semi-evergreen to shrubby forests. It is highly valued for its medicinal properties as dried pseudo-bulbs are important ingredients of several Ayurvedic preparations. In this study, adventitious shoot buds were induced from internodal explants of *M. acuminata* grown on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzyladenine (BA), kinetin (Kn), and thidiazuron (TDZ). Of the three cytokinins used, TDZ at 3 mg l^{-1} induced the highest frequency (82%) of organogenic explants. However, all responding explants produced only a single adventitious shoot irrespective of the type and concentration of the cytokinin. Adding 0.5 mg l^{-1} α naphthaleneacetic acid (NAA) to the medium enhanced adventitious shoot formation. In the presence of 3 mg l^{-1} TDZ and 0.5 mg l^{-1} NAA, frequency of organogenesis was 96% with a mean number of 6.1 shoots per explant. Prolonged culture or subculture on the same medium did not promote further shoot production. However, transfer of these cultures to MS medium supplemented with 3 mg l^{-1} TDZ and 0.5 mg l^{-1} NAA and various concentrations of different polyamines (PAs), including spermine, spermidine, and putrescine, significantly increased mean shoot number per explant. The highest frequency of shoot induction (100%) and mean shoot number per explant (14.6) was observed on MS medium with 3 mg l^{-1} TDZ, 0.5 mg l^{-1} NAA, and 0.4 mM spermidine. Regenerated shoots were excised and subcultured on an elongation medium consisting of MS medium with 3 mg l^{-1} BA. Moreover, the highest frequency of rooting (96%) and mean number of roots per shoot (3.3) was observed on MS medium with 4 mg l^{-1} indole-3-butyric acid (IBA) and 1.5 mg l^{-1} activated charcoal (AC). Almost 90% of rooted shoots were successfully acclimatized and established ex vitro.

Keywords Activated charcoal · Adventitious bud · *Malaxis acuminata* · Orchidaceae · Polyamines

Abbreviations

AC	Activated charcoal
BA	6-Benzyladenine
IBA	Indole-3-butyric acid
Kn	Kinetin
MS	Murashige and Skoog
NAA	α Naphthaleneacetic acid
PAs	Polyamines
TDZ	Thidiazuron

Introduction

Orchids are one of the largest and most diverse groups among angiosperms. According to one estimate, the family Orchidaceae includes 800 genera and 25,000 species (Stewart and Griffiths 1995). These ornamental plants are widely distributed, cultivated for their beautiful flowers, and are of economic importance. In addition to their ornamental value, orchids are also well known for their

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medicinal usage especially in the traditional folk medicine. The medicinal use of orchids was first reported by the Chinese (Bulpitt 2005). The Chinese pharmacopoeia, "the Sang Nung Pen Tsao Ching", illustrated *Dendrobium* as a source of tonic, astringent, analgesic, and anti-inflammatory compounds as far back as 200 BC (Singh and Tiwari 2007), and since the Vedic period in India (Singh and Tiwari 2007). Orchids are used in traditional medicine as they are rich in active compounds including several alkaloids (Okamoto et al. 1966; Lawler and Slaytor 1969; Elander et al. 1973; Nurhayati et al. 2009). In the Ayurvedic branch of traditional medicine, a group of eight drugs, known as "Ashtavarga", provide important ingredients for different types of tonics. Dried pseudo-bulbs of *Malaxis acuminata* serve as important sources of Astavarga utilized in the preparation of the Ayurvedic tonic 'Chyavanprash'. The latter is one of the most widely used Ayurvedic preparations for promoting human health and preventing disease (Uniyal 1975; Govindarajan et al. 2007).

Malaxis acuminata is a small, medium-sized terrestrial orchid, up to 30 cm in length, with pseudo-bulbs at the base, and with fibrous roots. Leaves have sheathing leaf base and new plants grow along the vicinity of the decaying mother plant. Flowers, in terminal racemes, are small, pale yellowish-green in color, but with a purple tinge.

Acute habitat destruction has resulted in the disappearance of this orchid from some areas of its natural habitat. Orchids are among the most vulnerable of plant families (Pridgeon 1996) with almost all orchid species forming a strong association with mycorrhizal fungi for development (Zettler 1997). Due to the economic importance of pseudobulbs of orchids, plants have been harvested excessively and beyond sustainable levels.

Tissue culture provides an alternate method for large-scale propagation of threatened and endangered plants, including orchid micropropagation using various explants (Tokuhara and Mii 2001; Chen et al. 2002a, 2005, 2009; Ket et al. 2004; Kauth et al. 2006; Thomas and Michael 2007). In this study, we report on an efficient shoot organogenesis system for *M. acuminata* using internodal explants.

Materials and methods

Plant material

Three- to four-year-old potted plants of *M. acuminata* growing at the Botanical Garden of St. Thomas College, Pala, were used as donor plants. Internodal stem segments, 1 cm in length, were excised from these donor plants and washed under running water for 30 min. Explants were then immersed in an aqueous solution of 4% (v/v) liquid detergent (Laboline; Qualigens, Mumbai, India) for 10 min, and rinsed three times with distilled water. Then, they were surface-sterilized with an aqueous solution of 0.1% (w/v) $HgC1_2$ for 8 min and rinsed three times with sterile distilled water.

Culture medium and growth conditions

Cut ends of internodal stem segments were trimmed before these were placed in culture tubes (one explant per tube) containing 12 ml Murashige and Skoog (1962) medium (MS) containing 100 mg l^{-1} (w/v) myo-inositol, 3% (w/v) sucrose (Qualigens), and solidified with 8 g l^{-1} agar (Bacteriological grade; Hi Media, Mumbai, India). This medium was supplemented with various plant growth regulators, including 1.0–4.0 mg l^{-1} 6-benzyladenine (BA), 1.0–4.0 mg l^{-1} thidiazuron (TDZ), 1.0–10.0 mg l^{-1} kinetin (Kn), either separately or in combination with 0.5 mg l^{-1} α naphthaleneacetic acid (NAA). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl prior to autoclaving. All medium-containing culture vessels were autoclaved at 104 kPa and 121°C for 20 min.

For each treatment, at least 24 explants were used, and all experiments were repeated three times. Each culture period lasted 8 weeks. All cultures were maintained at 25 ± 2°C, 70% relative humidity, and 16 h photoperiod of 35–50 µmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes (Phillips, Mumbai, India).

Data on number of organogenic explants and number of developing shoots per explant were recorded.

Effects of various polyamines on shoot organogenesis

The adventitious shoots from the media supplemented with BA (1–4 mg l^{-1}), TDZ (1–4 mg l^{-1}) or Kn (1–4 mg l^{-1}) in combination with NAA (0.5 mg l^{-1}) were randomly selected and transferred to MS medium containing 3 mg l^{-1} TDZ and 0.5 mg l^{-1} NAA, supplemented with various concentrations (0.2–1.0 mM) of polyamines, including spermine, spermidine, and putrescine.. Numbers of explants, replications, and culture conditions were as described above. The average adventitious shoot number was calculated at the time of culture as well as at the 4th and 8th weeks after culture.

Shoot elongation

Adventitious shoots were excised from explants and subcultured on MS medium supplemented with 3 mg l^{-1} BA for shoot elongation. All media were prepared as described above, and cultures were grown under conditions as described above.

Rooting of shoots, acclimatization, and field transfer

Individual shoots with two to three expanded leaves were transferred to half-strength MS medium supplemented with 2–8 mg l^{-1} of indole-3-butyric acid (IBA) or NAA (2–8 mg l^{-1}).

Rooted shoots, with 2–3 roots, were removed from culture tubes, and washed thoroughly in running tap water for 5–6 min to remove traces of agar. These were then planted in plastic cups (6 cm in diameter) containing a potting mixture of charcoal chips and soil (1:1). Plantlets were initially covered with a polythene sheet for 1 month to maintain high relative humidity (90%). These were irrigated every other day with half-strength MS liquid medium. The number of surviving plants was recorded after 12 weeks. All surviving acclimatized plants were eventually transferred to the field.

Data analysis

All data were subjected to analysis of variance using SAS, and means were compared using Duncan's multiple range test (Duncan 1955).

Results

When explants were cultured on MS basal medium supplemented with BA or TDZ or Kn alone, explants were organogenic, and each explant developed single shoots (Table 1). None of the explants incubated on a medium lacking any plant growth regulator (PGR) were organogenic. Among all three cytokinins tested, TDZ induced the highest frequency of shoot organogenesis, and this was followed by Kn. Among the different levels of TDZ evaluated, 3.0 mg l^{-1} TDZ resulted in 82% organogenic explants (Table 1). Explants produced a single small white protuberance after 4 weeks of culture. After another 4 weeks, this single protuberance directly differentiated into a well-defined shoot without any intervening callus or protocorm-like-body formation and was about 1.0 cm in length. Further elongation of the solitary shoot did not take place in the same medium. The explant turned dark or brown in color after 8 weeks of culture in most media tested.

When explants were grown on different cytokinin-containing media, but supplemented with 0.5 mg l^{-1} NAA, a higher organogenic response was observed on all PGR treatment combinations. The highest frequency of organogenic explants (96%) and number of adventitious shoots per explant (6.1) were observed on MS medium supplemented with 3 mg l^{-1} TDZ and 0.5 mg l^{-1} NAA (Table 1; Fig. 1a, b). Among Kn levels used, 2 mg l^{-1} Kn in combination with 0.5 mg l^{-1} NAA induced 88% of organogenic explants with 5.4 shoots per explant (Table 1).

Extended culture on the same medium or subculture on a fresh medium with the same composition did not improve shoot yield. Therefore, three polyamines (PAs) were tested to evaluate their role on adventitious shoot formation. Spermidine and putrescine produced more number of shoots than spermine (Table 2). All cultures were responded in four different concentrations of three PAs. However, the average shoot number per culture varied with the type and concentration of PA. Spermidine at 0.4 mM and putrescine at 0.4 and 0.8 mM concentrations gave a three-fold increase in shoot number after 8 weeks of culture (Table 2). Optimum response (9.8 and 14.6 shoots after 4 and 8 weeks of culture) was obtained on MS medium supplemented with TDZ (3 mg l^{-1}), NAA (0.5 mg l^{-1}) along with 0.4 mM spermidine (Table 2; Fig. 1c, d).

The whitish green shoots originated from the internodal explants did not show elongation and leaf development on shoot induction medium, furthermore the average shoot length remained almost similar to earlier experiments. Therefore, for further elongation of shoots, the shoot clumps were detached from the explant and transferred to MS medium supplemented with 3 mg l^{-1} BA. On this medium, the shoots attained an average height of 1.8 cm with expanded leaves in 8 weeks (Fig. 1e).

For rooting, the shoots were transferred to MS medium containing IBA (2–8 mg l^{-1}) and NAA (2–8 mg l^{-1}). However, there was no rooting on this medium despite reducing the concentration of medium to half strength. Thus, 1.5 mg l^{-1} activated charcoal (AC) was added to promote rooting. The shoots failed to root on lower concentrations (0.2–1.0 mg l^{-1}) of IBA and NAA even in the presence of AC (data not shown). However, in higher concentrations (2.0–8.0 mg l^{-1}), rooting efficiency was significantly improved in presence of AC. Maximum response (96%) was observed on MS medium supplemented with 4 mg l^{-1} IBA and 1.5 mg l^{-1} AC (Table 3; Fig. 1f). On this medium, an average number of 3.3 roots per shoot were observed after 8 weeks. Comparatively, IBA gave better result than NAA in terms of percent cultures responding and number of roots per shoot (Table 3).

The plants were transferred to the greenhouse after 4 weeks, where they have acclimatized. The survival percentage was 90% after 12 weeks in the greenhouse. No phenotypic variation was observed among the in vitro raised plants.

Discussion

The objective of this study was to develop an efficient in vitro multiple shoot induction system, which will allow

Table 1 Effects of various concentrations of BA, TDZ, and Kn alone or in combination with NAA on adventitious shoot formation from cultured internodal explants of *M. acuminata* 8 weeks after culture

Plant growth regulators (mg l^{-1})				Frequency of organogenic explants (%)	Mean no. of shoots per explant[a]	Mean shoot length (cm)[a]
BA	TDZ	Kn	NAA			
0.0	0.0	0.0	0.0	0.0	0.0±	0.0
1.0	–	–	–	55 e	1.0 ± 0.0 f	0.8 ± 0.05 b
2.0	–	–	–	63 d	1.0 ± 0.0 f	0.9 ± 0.07 b
3.0	–	–	–	68 d	1.0 ± 0.0 f	0.7 ± 0.08 b
4.0	–	–	–	65 d	1.0 ± 0.0 f	0.8 ± 0.05 b
–	1.0	–	–	66 d	1.0 ± 0.0 f	0.9 ± 0.07 b
–	2.0	–	–	78 c	1.0 ± 0.0 f	1.2 ± 0.06 a
–	3.0	–	–	82 b	1.0 ± 0.0 f	1.1 ± 0.07 a
–	4.0	–	–	72 c	1.0 ± 0.0 f	1.2 ± 0.08 a
–	–	1.0	–	62 d	1.0 ± 0.0 f	1.1 ± 0.06 a
–	–	2.0	–	65 d	1.0 ± 0.0 f	1.0 ± 0.07 a
–	–	3.0	–	71 c	1.0 ± 0.0 f	0.9 ± 0.08 b
–	–	4.0	–	67 d	1.0 ± 0.0 f	0.9 ± 0.06 b
1.0	–	–	0.5	80 b	2.3 ± 0.4 e	0.7 ± 0.07 b
2.0	–	–	0.5	84 b	2.7 ± 0.2 e	0.8 ± 0.07 b
3.0	–	–	0.5	87 b	3.1 ± 0.3 d	0.7 ± 0.08 b
4.0	–	–	0.5	78 c	2.9 ± 0.3 e	0.7 ± 0.07 b
–	1.0	–	0.5	81 b	2.7 ± 0.4 e	0.9 ± 0.06 b
–	2.0	–	0.5	87 b	4.4 ± 0.2 c	0.8 ± 0.05 b
–	3.0	–	0.5	96 a	6.1 ± 0.2 a	0.7 ± 0.07 b
–	4.0	–	0.5	88 b	5.8 ± 0.4 b	0.8 ± 0.06 b
–	–	1.0	0.5	74 c	3.5 ± 0.3 d	0.9 ± 0.08 b
–	–	2.0	0.5	88 b	5.4 ± 0.6 b	0.8 ± 0.05 b
–	–	3.0	0.5	83 b	5.1 ± 0.5 b	0.9 ± 0.04 b
–	–	4.0	0.5	72 c	4.6 ± 0.4 c	0.7 ± 0.06 b

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

[a] Values correspond to means (±SE) of three independent experiments. At least 24 cultures were used for each experiment.

large-scale multiplication of the primary induced sterile shoots. Since the plants are medicinally useful and threatened, the use of an efficient micropropagation system as a means to multiply for controlled production of the desired plants will take the pressure off the wild populations.

In our work, TDZ was more beneficial in inducing shoots than other cytokinins. The induction rate varied with type and concentration of growth regulators. Of the various auxin–cytokinin combinations meant for shoot induction, 3 mg l^{-1} TDZ and 0.5 mg l^{-1} NAA induced maximum response with 96% cultures responding with an average number of 6.1 shoots per explant. Thus, in this study, TDZ played a central role for shoot induction in *M. acuminata*. After the first report of cytokinin-like activity of TDZ by Mok et al. (1982), TDZ has been successfully used to induce adventitious shoot formation in numerous systems, particularly woody plants, and is reported to be more efficient than purine-type cytokinins (BA or Kn) even at extremely low concentrations (Huetteman and Preece 1993).

TDZ has been effectively utilized in orchids to induce direct somatic embryogenesis in *Oncidium* (Chen et al. 1999; Chen and Chang 2001), shoot regeneration in *Phalaenopsis*, *Doritaenopsis*, and epiphytic *Cymbidium* (Ernst 1994; Chen and Piluek 1995; Nayak et al. 1997), embryogenic callus induction in *Cymbidium ensifolium* and *Oncidium* sp. (Chang and Chang 1998; Chen and Chang 2000a, b), induction of protocorm like bodies (PLB) from flower stalk of *Epidendrum radicans* (Chen et al. 2002b), and multiple shoot induction in *Rhynchostylis retusa* (Thomas and Michael 2007).

Our investigation confirmed the positive role of PAs in inducing adventitious shoot induction. Spermidine and putrescine produced enhanced results compared with spermine when tested with TDZ and NAA. The significantly high shoot number was observed when spermidine at

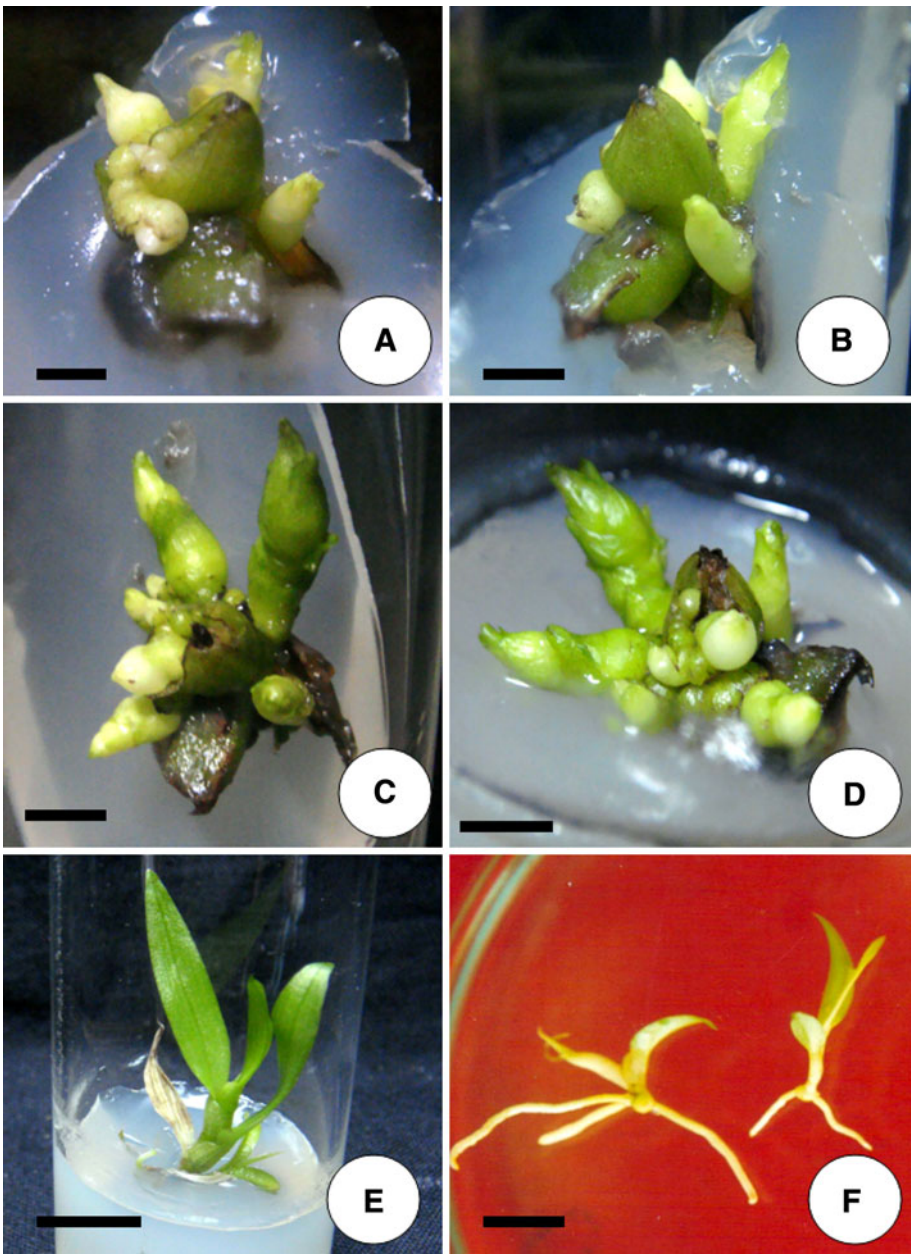

Fig. 1 Various stages of adventitious shoot bud induction, shoot elongation and rooting of shoots from internodal explants of *M. acuminata*. **a** Adventitious shoot induction on explants grown on MS medium supplemented with 3 mg l^{-1} TDZ and 0.5 mg l^{-1} NAA after 4 weeks of culture. An average number of about six shoots were produced per explant. *Bar* 0.8 cm. **b** Same as in (**a**) after 8 weeks of culture. *Bar* 0.8 cm. **c** Adventitious shoot formation on MS medium fortified with 3 mg l^{-1} TDZ, 0.5 mg l^{-1} NAA and 0.4 mM spermidine 4 weeks after subculture from MS with 3 mg l^{-1} TDZ and 0.5 mg l^{-1} NAA. A mean number of 14.6 shoots was observed. *Bar* 1 cm. **d** Same as in (**c**) 8 weeks after culture. Adventitious shoots have developed further. *Bar* 1 cm. **e** Shoots on elongation medium which consists of MS medium supplemented with 3 mg l^{-1} BA. Shoots were 1.8 cm in 8 weeks. *Bar* 0.8 cm. **e** Rooted shoots taken out from MS medium supplemented with 4 mg l^{-1} IBA and 1.5 g l^{-1} AC 8 weeks after culture. *Bar* 1 cm

0.4 mM and putrescine at 0.4 and 0.8 mM concentrations were employed in the medium.

Polyamines (PAs) are molecules that are responsible for different plant developmental processes (Silveira et al. 2006; Tun et al. 2006). They control several cellular processes including DNA replication, cell division, protein synthesis, flower development, in vitro flower induction, fruit development, senescence, abiotic and biotic stress responses, and secondary metabolism (Kumar et al. 1997; Bagni and Tassoni 2001; Bais and Ravishankar 2002; Kuznetsov et al. 2006; Tun et al. 2006). PAs are extensively used for various purposes in plant tissue culture including somatic embryogenesis (Feirer 1995; Minocha et al. 1999; Kevers et al. 2002; Rajesh et al. 2003; Bertoldi et al. 2004; Silveira et al. 2006; Santa-Catarina et al. 2007; Venkatachalam and Bhagyalakshmi 2008) root induction (Biondi et al. 1990; Heloir et al. 1996; Grigoriadou et al. 2002; Couee et al. 2004), androgenesis, and gynogenesis (Tiainen 1992; Rajyalakshmi et al. 1995; Martinez et al. 2000; Ashok Kumar et al. 2004; Chiancone et al. 2006). Similarly, polyamine induced callus regeneration in sugar beet (Hagege et al. 1994) and direct shoot regeneration in *Brassica campestris* (Chi et al. 1994) and Chinese radish (Pua et al. 1996) has also been reported. In orchids like *Vanilla planifolia* and *Dendrobium*, PAs promoted in vitro propagation (Thyagi et al. 2001; Saiprasad et al. 2004).

Table 2 Effects of different concentrations of various polyamines on adventitious shoot induction from internodal explants. The internodal explant with an average number varying from 5.3 to 5.8 shoots from various growth regulator combinations were subcultured on polyamine-containing media

Polyamines (mM)			Mean no. of shoots per explant at the time of culture[a]	Mean no. of shoots per explant after 4 weeks of culture[a]	Mean no. of shoots per explant after 8 weeks of culture[a]	Mean shoot length (cm)[a]
Spermine	Spermidine	Putrescine				
0.0	0.0	0.0	5.6 ± 0.3 a	5.6 ± 0.0 d	5.6 ± 0.0 f	0.6 ± 0.0 b
0.2	–	–	5.6 ± 0.2 a	8.3 ± 0.3 b	10.1 ± 0.5 e	0.6 ± 0.03 b
0.4	–	–	5.8 ± 0.4 a	8.7 ± 0.6 b	11.3 ± 0.3 d	0.8 ± 0.02 b
0.8	–	–	5.5 ± 0.2 a	8.6 ± 0.4 b	10.6 ± 0.6 e	0.5 ± 0.04 b
1.0	–	–	5.6 ± 0.5 a	8.2 ± 0.8 b	10.2 ± 0.4 e	0.7 ± 0.03 b
–	0.2	–	5.8 ± 0.4 a	7.3 ± 0.7 c	11.3 ± 0.3 d	0.8 ± 0.01 b
–	0.4	–	5.4 ± 0.3 a	9.8 ± 0.4 a	14.6 ± 0.5 a	0.6 ± 0.03 b
–	0.8	–	5.5 ± 0.4 a	9.4 ± 0.3 a	13.7 ± 0.4 b	0.5 ± 0.04 b
–	1.0	–	5.3 ± 0.4 a	7.8 ± 0.2 c	11.8 ± 0.7 d	0.6 ± 0.03 b
–	–	0.2	5.6 ± 0.3 a	8.3 ± 0.5 b	12.7 ± 0.6 c	0.8 ± 0.05 b
–	–	0.4	5.3 ± 0.2 a	9.6 ± 0.4 a	14.4 ± 0.5 a	1.1 ± 0.03 a
–	–	0.8	5.8 ± 0.2 a	9.1 ± 0.4 a	14.2 ± 0.4 a	0.6 ± 0.05 b
–	–	1.0	5.6 ± 0.4 a	8.7 ± 0.5 b	12.3 ± 0.6 c	0.7 ± 0.06 b

Values correspond to means (±SE) of three independent experiments. At least 24 cultures were used for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

[a] For all treatment combinations tested, 100% explants were organogenic

Table 3 Effects of different concentrations of IBA and NAA in combination with 1.5 g l^{-1} activated charcoal (AC) on rooting of *M. acuminata* shoots after 60 days of culture

Auxin (mg l^{-1})		Frequency of shoots with roots[a]	Mean no. of roots per shoot[a]	Mean root length (cm)[a]
IBA	NAA			
0.0	0.0	0.0	0.0	0.0
2.0	–	87 c	2.3 ± 0.2 b	1.2 ± 0.06 b
4.0	–	96 a	3.3 ± 0.1 a	1.9 ± 0.04 a
6.0	–	93 b	2.8 ± 0.3 b	1.5 ± 0.07 b
8.0	–	90 b	2.4 ± 0.2 b	1.3 ± 0.02 b
–	2.0	74 d	2.2 ± 0.3 b	1.6 ± 0.06 b
–	4.0	81 c	2.5 ± 0.2 b	1.4 ± 0.05 b
–	6.0	87 c	2.6 ± 0.1 b	1.5 ± 0.08 b
–	8.0	83 c	2.1 ± 0.3 b	1.4 ± 0.03 b

At least 24 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

[a] The values represent the means (±SE) of three independent experiments.

Root induction was observed only in the presence of AC irrespective of auxins used. AC at 1.5 mg l^{-1} was optimum for root induction with IBA (2.0–8.0 mg l^{-1}) and NAA (2.0–8.0 mg l^{-1}). In orchids like *Cypripedium flavum*, 100% rooting was observed on medium containing AC (0.6 g l^{-1}) alone. This is probably due to the partial darkness created by the AC in the medium which is similar to the underground environment of *C. flavum* habitats (Yan et al. 2006). AC-stimulated rooting has also been reported in other orchids like *Renanthera imschootiana* (Seeni and Latha 1992), *Anoectochilus formosanus* (Ket et al. 2004), *Cymbedium faberi* (Chen et al. 2005), and *Dendrobium* hybrid (Martin and Madassery 2006).

In conclusion, this simple and efficient method for micropropagating large number of plantlets via internodal adventitious shoots could be used for large-scale propagation and ex situ conservation of the medicinal terrestrial orchid *M. acuminata*.

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