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High-frequency Regeneration of Shoots from Cotyledon and Leaf Explants of a Medicinal Cucurbit, *Mukia maderaspatana* (L.) M.J. Roem.

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ABSTRACT

The purpose of this study was to develop an efficient protocol for *in vitro* organogenesis from cotyledon and leaf explants of *Mukia maderaspatana* (Linn.) M. J. Roem., a medicinal member of the family Cucurbitaceae. Cotyledon explants isolated from *in vitro* germinated seedlings (5-6 days old) were cultured on Murashige and Skoog (MS) medium containing different concentrations of 6-benzyladenine (BA; 0.89, 1.78, 2.22, 3.11, 4.40 and 6.62 μM) alone, or in combination with indole-3-acetic acid (IAA; 0.57 μM). Cotyledon explants cultured on medium containing BA (2.22 μM) and IAA (0.57 μM) induced a significantly high number of multiple shoots (9.00 ± 0.60) with increased mean shoot length (2.70 ± 0.10) within 5 week of culture. Leaf segments from *in vitro* grown plants (20 days-old) were cultured on MS medium with different concentrations of BA (0.22, 0.44, 0.89, 1.78, 2.22, 3.11 and 4.40 μM) alone, or together with IAA (0.57 μM). Maximum number of shoots (10 ± 0.75) with increased mean shoot length (2.90 ± 0.12) were obtained directly from leaf explants (without intervening callus phase) using a combination of BA (0.89 μM) and IAA (0.57 μM) within 5 weeks of culture. Inclusion of IAA to MS medium with BA triggered 80% of regeneration from leaf and cotyledon explants. Elongation of regenerated shoots occurred when cotyledon cultures (3 weeks-old) were transferred to MS basal medium. Leaf cultures with emerging shoots were sub-cultured onto the same treatment medium for further elongation. The elongated shoots (2-3 cm) were excised and rooted on MS medium supplemented with IBA (2.46 μM). Rooted plants were acclimatized in the greenhouse with a 70% survival rate.

Keywords: BA, hardening, IAA, multiple shoots, traditional medicinal
Abbreviations: BA, 6-benzyladenine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **MS**, Murashige and Skoog

INTRODUCTION

Cucurbitaceae is an economically important family of plants, with species commercially cultivated in tropical and subtropical regions. Among cucurbit species *Mukia maderaspatana* syn. *Melothria maderaspatana* or *Cucumis maderaspatana* is a medicinally important climber with pubescens shoots. This plant is distributed throughout India. Whole plant parts are used in traditional medicine for curing various disorders. It is used as an antihelmentic, laxative, anesthetic, sedative, bronchitis, expectorant, astringent, stimulant, and for the treatment of bronchitis, elephantiasis, piles, and ulcers (Parekh and Chanda 2007). The plant is also used in preparation to treat chronic cough and cold (Chellaiah *et al.* 2006). Leaf paste is used to treat scabies and ringworm infection (Anitha *et al.* 2008). Certain traditional medical practitioners also use the leaf-tea of this plant for the alleviation of jaundice (Attygalle *et al*. 1952; Jayaweera 1982). Decoctions of leaves from this plant have been used by Siddha practitioners (Marundugalin 2001) in Tamil Nadu for the treatment of hypertension. Plant leaf extract has also been shown to have hepatoprotective and immunomodulatory effects (Thabrew *et al*. 1991, 1995) and antiarthritic activity properties (Ramakrishanamacharya *et al*. 1996). The pharmacological significance of this plant is mainly because of various bioactive compounds such as glycosides, triterpenoid saponins, spinosterol and dihydrospinasterol (Sinha *et al*. 1996, 1997). *M. maderaspatana* grows in forests and there is no organized propagation available for this plant species. Therefore, it is essential to take necessary steps to propagate the plant for its conservation. *In vitro* propagation can be used as an effective strategy for germplasm conservation and multiplication of this important plant species.

Application of *in vitro* regeneration techniques for cucurbits has been reported from cotyledon nodes (Gambley and Dodd 1991), leaves (Mishra and Bhatnagar 1995), leaves and cotyledons (Stippe *et al.* 2001; Kathiravan *et al*. 2006), and anthers (Kumar *et al.* 2003). On the other hand, the organogenic route for plant regeneration from callus cultures is reported in *C. pepo* (Jelaska *et al.* 1985). An organogenic pathway bypassing callus formation has also been reported from seedling-derived cotyledons in cucurbits (Rakoczy-Trojanowska and Malepszy 1989; Ananthakrishnan *et al.* 2003). Plant biotechnology appears to be a viable option for the improvement of cucurbit species by means of plant tissue culture and genetic transformation. The primary step in this approach will be to establish an efficient *in vitro* plant regeneration system using leaves and/or cotyledons as the most favoured explant source for tissue culture and genetic transformation studies. To date no reports are available that describe direct organogenesis in *M. maderaspatana.* We report for the first time an efficient and reproducible protocol for multiple shoot induction from cotyledon and leaf explants of *M. maderaspatana.*

MATERIALS AND METHODS

Seed collection and preparation

Mukia maderaspatana seeds were collected from plants grown under field conditions in the departmental garden, identified by Prof. V.S. Raju, Kakatiya University, Warangal, India. The seeds were treated with 5% Tween-20 for 5 min followed by 3-4 rinses

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and soaking for 8 h duration, both in sterile distilled water (SDW). The soaked seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 4-5 min followed by 4-5 rinses with SDW. The surface-sterilized seeds were inoculated in 200 ml glass jars each containing 50 ml of MS (Murashige and Skoog 1962) basal medium for germination at 27°C under white fluorescent light (40-60 µmol $m^{-2}s^{-1}$; IS 2416 L 7434877, two bulbs Phillips, India) (65 µE/m^2/s) with a 16-h photoperiod.

Cotyledon and leaf regeneration studies

Cotyledons collected from *in vitro*-grown seedlings (5-6 days-old) were cut into 1.0 cm^2 explants. In order to study the effect of plant growth regulators (PGRs) on shoot induction, cotyledon segments (one segment per tube) were cultured in tubes (150 × 25 mm) containing MS basal medium, supplemented with 6-benzyladenine (BA) (0.89, 1.78, 2.22, 3.11, 4.40 and 6.62 µM) alone, or in combination with indole-3-acetic acid (IAA; 0.57 µM). The explants were cultured for 3 weeks on media followed by transfer for elongation of shoots onto MS basal medium. In another set of experiments, leaves excised from *in vitro*-grown plants (20-days old) were cut into explants 1.0 cm^2 in size. Explants were cultured on MS medium supplemented with BA (0.22, 0.44, 0.89, 1.78, 2.22, 3.11 and 4.40 µM) alone, or in combination with IAA (0.57 µM). Following 3 weeks of culture on regeneration medium, the explants were transferred once onto the same treatment medium for further elongation of shoots then transferred to rooting medium (MS medium supplemented with indole-3-butyric acid, IBA).

All elongated individual microshoots (2.0-2.2 cm) with 2-3 leaves were excised and rooted on MS medium with indole-3-butyric acid (IBA; 2.46, 4.90 and 7.56 µM). Shoots 4.0–4.5 cm in length with 4-5 leaves were obtained 2 weeks after culture on rooting medium. These developed shoots were removed from the culture tubes and washed once or twice in running tap water to remove agar and medium constituents followed by transfer into plastic pots containing autoclaved sand and top soil (1: 1) mixture. The potted plants were hardened in the greenhouse (28°C maintained during the daytime, 16-18°C at night; 60 µmol $m^{-2}s^{-1}$ light intensity). For regeneration and rooting studies, MS media with 2% sucrose (Hi-media, India) and 0.8% (w/v) agar (Hi-media) were used. All media pH was adjusted to 5.8 with 0.1 N NaOH before autoclaving at 121°C for 15 min. Each culture tube (150 × 25 mm) containing 20 ml of medium was inoculated with a single explant and plugged with non-adsorbent cotton wrapped in two layers of cheese cloth.

Data pertaining to responding cultures (i.e., percent of explants exhibiting shoot development), and number and/or length of shoot was recorded 5 weeks after the beginning of culture. All data were statistically analyzed using Duncan's multiple range test (DMRT) for mean comparison ($P \leq 0.05$). Altogether, 10 explants were used in each of two replicates for each treatment and the experiment was repeated twice.

RESULTS

Shoot regeneration from cotyledon

The medium for cotyledon regeneration contained different concentrations of BA alone, or BA in combination with IAA. Under both these conditions of PGRs, the shoot formation was restricted to the proximal cut edge of the explants. Shoot regeneration accompanied callus formation when BA alone was used as the PGR supplement. Significantly high number (6.00 ± 0.50) of shoots were obtained at 2.22 µM BA with the mean length of 2.20 ± 0.08. An increase or decrease in the BA level (1.78, 3.11 and 4.40 µM) caused a reduction in the per cent responding cultures and mean shoot number (**Table 1**). BA at 0.89 and 6.62 µM resulted in callus formation without shoot induction.

Medium containing BA (2.22 µM) + IAA (0.57 µM) was superior in promoting high frequency shoot regeneration and multiple shoot induction (9.00 ± 0.60) with an average shoot length of 2.70 ± 0.10.

This condition led to a morphogenic response and plant regeneration without callus induction (**Fig. 1A, 1B**). Multiple shoots were also regenerated from cotyledon explants at low (0.89, 1.78 µM) or high (3.11, 4.40, 6.62 µM) concentrations of BA with IAA (0.57 µM). However, their number remained low as compared to optimal levels of BA (2.22 µM) and IAA (0.57 µM) (**Table 1**). A drastic reduction in the percentage of responding cultures was observed at these levels of BA + IAA (**Table 1**). An increased in BA (4.40, 6.62 µM) concentration with IAA (0.57 µM) showed a significant decline in the shoot number along with intervening callus phase. Three weeks after culture on regeneration medium, the cotyledon cultures were transferred to MS basal medium. Shoots elongated on this medium within 2 weeks of culture.

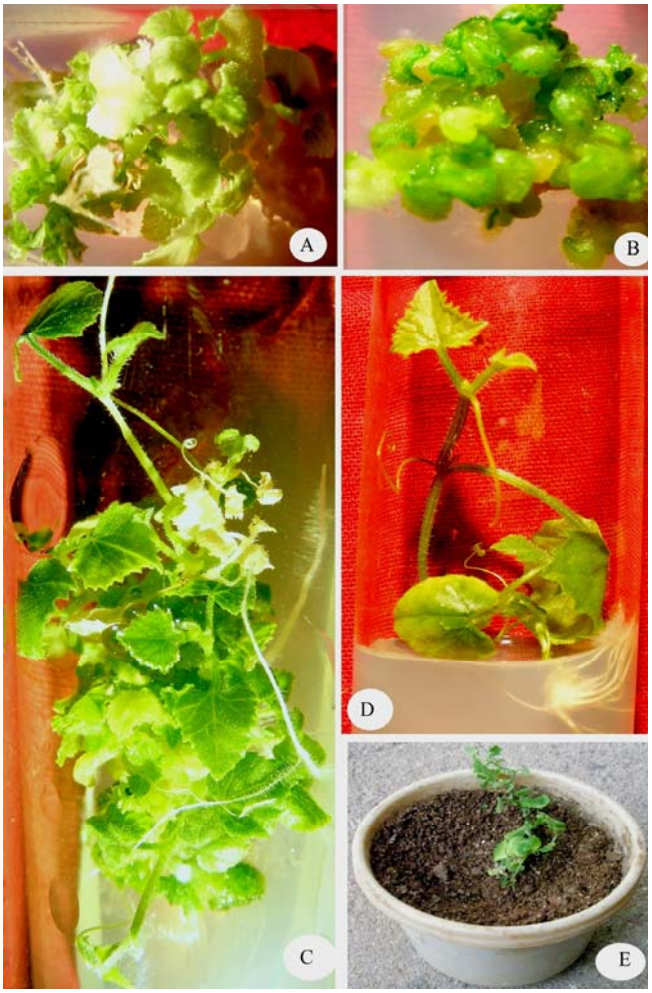

Fig. 1 The effects of plant growth regulator (BA or BA + IAA) on multiple shoot induction in *Mukia maderaspatana* leaf and cotyledon explants. (**A**) Direct leaf regeneration on MS medium containing, BA (0.89 µM) + IAA (0.57 µM) (after 3 weeks). (**B**) Regenerating adventitious shoots from cotyledon (proximal portion) at a concentration of BA at (2.22 µM) + IAA at (0.57 µM) (after 3 weeks). (**C**) Elongation of leaf culture microshoots after 5 weeks of subculture onto the same medium (total of 3 ± 2 weeks). (**D**) Rooting of microshoots on MS medium supplemented with IBA (2.46 µM) after 2 weeks. (**E**) Acclimatization of regenerated shoots under greenhouse conditions.

Shoot regeneration from leaves

Leaf explants excised from *in vitro* grown plants were cultured on MS medium fortified with BA or BA + IAA. Shoot regeneration was achieved on media with BA (0.44, 0.89, and 1.78 µM) as the sole cytokinin, or BA (0.44, 0.89, 1.78, 2.22 and 3.11 µM) in combination with the auxin IAA (0.57 µM). The combination of BA and IAA was superior in promoting shoot development in *M. maderaspatana* than individual use of BA. The leaf cultures showed efficient regeneration response on medium containing BA (0.89 µM)

Table 1 Effect of BA or BA + IAA on multiple shoot induction from cotyledon explants of *Mukia maderaspatana*.

Hormone (µM)	% response	Morphogenic response	Mean number of shoots/explant ± S.E.	Mean length of shoot (cm ± S.E.)
BA				
0.89	20	C	0.00 ± 0.0 a	0.00 ± 0.00 a
1.78	30	S ± C	4.00 ± 0.31 d	1.80 ± 0.19 f
2.22	60	S ± C	6.00 ± 0.50 e	2.20 ± 0.08 g
3.11	50	S ± C	5.00 ± 0.42 e	1.40 ± 0.06 b
4.4	55	S ± C	2.00 ± 0.18 b	1.00 ± 0.06 b
6.62	45	C	0.00 ± 0.00 a	0.00 ± 0.00 a
BA + IAA				
0.89 ± 0.57	20	S	2.00 ± 0.13 b	1.70 ± 0.10 d
1.78 ± 0.57	50	S	5.00 ± 0.37 e	1.80 ± 0.07 f
2.22 ± 0.57	80	S	9.00 ± 0.60 f	2.70 ± 0.10 g
3.11 ± 0.57	60	S	5.00 ± 0.44 e	2.10 ± 0.70 g
4.40 ± 0.57	70	S ± C	3.00 ± 0.24 c	1.90 ± 0.10 f
6.62 ± 0.57	55	S ± C	2.00 ± 0.18 b	1.50 ± 0.05 c

s-shoot; c-callus
Values are mean of 40 explants ± S.E.
In each column mean followed by same letter were not significantly different (P≤0.05) according to Duncan's multiple range test.

Table 2 Effect of BA or BA + IAA on multiple shoot induction from leaf explants of *Mukia maderaspatana*.

Hormone (µM)	% response	Morphogenic response	Mean number of shoots/explant ± S.E.	Mean length of shoot (cm ± S.E.)
BA				
0.22	55	C	0.00 ± 0.00 a	0.00 ± 0.00 a
0.44	60	S ± C	3.00 ± 0.27 b	1.80 ± 0.06 c
0.89	75	S ± C	4.00 ± 0.35 c	2.20 ± 0.06 e
1.78	70	S ± C	3.00 ± 0.24 b	1.40 ± 0.06 b
2.22	65	C	0.00 ± 0.00 a	0.00 ± 0.00 a
3.11	50	C	0.00 ± 0.00 a	0.00 ± 0.00 a
4.4	60	C	0.00 ± 0.00 a	0.00 ± 0.00 a
BA + IAA				
0.22 ± 0.57	55	C	0.00 ± 0.00 a	0.00 ± 0.00 a
0.44 ± 0.57	65	S	5.00 ± 0.43 d	2.20 ± 0.08 e
0.89 ± 0.57	80	S	10.00 ± 0.75 f	2.90 ± 0.12 f
1.78 ± 0.57	70	S	6.00 ± 0.48 e	2.40 ± 0.11 e
2.22 ± 0.57	65	S	5.00 ± 0.44 d	2.00 ± 0.09 d
3.11 ± 0.57	60	S ± C	4.00 ± 0.33 c	2.20 ± 0.08 e
6.62 ± 0.57	55	C	0.00 ± 0.00 a	0.00 ± 0.00 a

s-shoot; c-callus
Values are mean of 40 explants ± S.E.
In each column mean followed by same letter were not significantly different (P≤0.05) according to Duncan's multiple range test.

with IAA (0.57 µM) (**Fig. 1A**). At these hormone levels, maximum number of shoots (10.00 ± 0.75) with an increased mean shoot length (2.90 ± 0.12) were obtained (**Fig. 1C**) after 2 weeks.

Leaf explants placed on media with BA + IAA (0.22, 6.62 ± 0.57 µM) produced callus tissue without shoot formation. We observed that a combination of BA (3.11 µM) with IAA (0.57 µM) induced shoot development along with callus formation (**Table 2**). Fewer shoots developed on media with BA alone as compared to optimal levels of BA (0.89 µM) + IAA (0.57 µM). Leaf cultures (3 weeks-old) that showed a regeneration response were subcultured onto medium with identical PGR supplements. Subsequently, further differentiation and elongation into well developed shoots was obtained 2 weeks after culture on these media. Well developed shoots were obtained after 5 weeks of culture. For leaf and cotyledon explants, regeneration was completed within 3 weeks and no new shoots emerged after this time period.

Individual shoots developed healthy roots after 10-12 days of culture on MS medium supplemented with IBA (2.46 µM) (**Fig. 1D**). Shoots derived either from cotyledon or leaf explants: both rooted to a similar extent with 75-80% rooting efficiency. Rooted plants were hardened in a 1: 1 mixture of sand and top soil followed by transfer to the greenhouse (**Fig. 1E**). The survival rate of the plants under greenhouse conditions was 70%.

DISCUSSION

In this study high frequency shoot regeneration was established from cotyledon and leaf explants of *M. maderaspatana*. The specific regions of cotyledons are shown to be important for morphogenesis induction of shoots in *Cucurbitaceae* species (Compton 2000; Ananthakrishnan *et al.* 2003). Our results are consistent with these reports wherein organogenic competence was concentrated at the proximal region of cotyledon explants of *M. maderaspatana*. The presence of a PGR supplement in the regeneration medium is important for adventitious bud development; and the cytokinin BA was critical for shoot initiation from cotyledon explants of *M. maderaspatana*. Similar results demonstrating the role of BA in regeneration of cotyledon explants were obtained in watermelon (Dong and Jia 1991) and *C. maxima* (Lee *et al.* 2003). Superiority of BA compared to other cytokinins for adventitious bud induction was reported in *Cucumis melo* L. (Ficcadenti and Rotino 1995; Sing *et al.* 1996). Our results also indicate that within the range evaluated BA (0.89-6.62 µM) showed significant difference for the number of developing shoots. BA alone at low (0.89 µM) concentration failed to induce shoot proliferation from cotyledon explants. The inclusion of IAA together with BA in the medium induced more shoots from cotyledon explants of *M. maderaspatana.* Thus, the presence of IAA in combination with BA appeared to be critical in evoking more shoots from cotyledon explants than the presence of BA alone (2.22 µM). Similar observations, where a combination of cytokinin (BA) and auxin (IAA) showed a positive influence on shoot regeneration ability, were reported

with other plant species like pepper (Rosa and Angles 1991), tomato cv. 'Microtom' (Venugopal Rao *et al.* 2005) and *Scoparia dulcis* (Aileni *et al*. 2008). This suggests that a balance between cytokinin and auxin concentration can govern the *in vitro* development pattern in a wide variety of species. The shoot initials produced from cotyledon explants were subcultured onto MS basal medium for further development and elongation. Similar observations were previously reported in cucumber (Selvaraj *et al.* 2007). BA (0.89 µM) was effective in inducing shoot regeneration at a low concentration in conjunction with IAA (0.57 µM). In conclusion, the regeneration and development of shoots from cotyledons of *M. maderaspatana* were improved by optimizing the cytokinin (BA) and auxin (IAA) combination. The study described here was also undertaken to examine the response of mature leaf cultures for direct regeneration in *M. maderaspatana.* The synergistic action of BA and IAA resulted in successful leaf regeneration. Hence a proper combination and balance of PGRs (auxins and cytokinins) evoked a regeneration response from leaf cultures of *M. maderaspatana*. Our data on leaf regeneration show that BA and IAA even at concentrations as low as 0.44 and 0.57 µM respectively show a positive effect on *in vitro* leaf regeneration. In *M. maderaspatana*, IAA behaved like a critical regulator to further promote *in vitro* shoot bud development. This study also makes clear the interaction between PGRs and determines whether or not BA and IAA have an additive relationship that can support *in vitro* regeneration.

In conclusion, the objective of the present work was to develop an efficient organogenesis protocol for *M. maderaspatana.* We demonstrated the use of cotyledon and leaf explants for direct regeneration of *M. maderaspatana*, a medicinal member of the Cucurbitaceae family. The protocol is efficient, reproducible and 9-10 microshoots from a single explant can be obtained. This organogenesis pattern was dependent on the use of a combination of auxin and cytokinin in the induction medium. This protocol ensures rapid clonal multiplication of *M. maderaspatana*.

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