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Original Research Article

An efficient protocol for micropropagation and genetic stability analysis of *Melia dubia* Cav. - an important multipurpose tree

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A B S T R A C T

Melia dubia has increasing demand industrially and economically because of its potential use in bioenergy applications and high timber quality. In order to meet its increasing demand, the present study was aimed to develop an efficient protocol for micropropagation of *Melia dubia* Cav. through axillary shoot proliferation from the explants of mature trees and assessment of genetic stability of tissue culture raised plants using RAPD markers. Highest (90%) bud break was obtained in Murashige and Skoog (MS) medium supplemented with additives, 2.22 µM 6- Benzyl amino purine (BAP) and 0.54 µM α-Naphthalene acetic acid (NAA) whereas, 2.22 µM BAP alone was found to be the most suitable growth regulator for achieving shoot multiplication at a rate of 4.92 fold every 4 weeks. Half strength MS basal medium with 2.47 µM Indole-3-butyric acid (IBA) induced 98% rooting. The survival rate during hardening was 90% and there was no genetic variation in the plants when compared to mother plant. In conclusion, the protocol developed here can be successfully utilized for industrial mass clonal propagation and the conservation of the species.

K e y w o r d s

Meliaceae, Axillary Shoot, proliferation Shoot, multiplication Rooting, Genetic fidelity

Introduction

Melia dubia Cav. (Meliaceae), an important multipurpose tree, bears clean cylindrical bole and usually attains 6 -12 m in height with big branches. The species is native to southern Asia (India-Pakistan-Iran) and has been introduced to South Africa, Middle East, America (Bermuda, Brazil and Argentina), Australia, Southeast (SE) Asia-Pacific islands and southern Europe. This is one of the three important indigenous species identified for *ex situ* conservation under the Medicinal Plant Project in Sri Lanka (Munasinghe, 2003).

Due to its multipurpose uses like bioenergy production, paper and pulp manufacturing, furniture making, building constructions, making musical instruments etc., it is gaining more popularity and is in high demand (Mandang, 2003; Suprapti et al., 2004; Parthiban et al., 2009; Chinnaraj et al., 2011). In addition, the extract from

different parts of the *M. dubia* plant are known to have antiviral, antibacterial, antifungal, antidiabetic, antineoplastic, antihelmintic and antileprosy properties (Kiritkar and Basu, 1999; Pettit, 2002; Nagalakshmi et al., 2003; Vijayan et al., 2004; Gerige and Ramjaneyulu, 2007; Susheela et al., 2008; Sukumaram and Raj, 2010; Sharma and Arya, 2011).

For this reason *M. dubia* trees growing naturally have been indiscriminately logged and resulted in significant decline in its population. Conventionally, *M. dubia* is propagated through seeds, which have very poor (14%-34.3%) germination rates because of hard stony seed coat, which makes it difficult to germinate without any treatment (Nair et al., 2005; Manjunatha, 2007; Anand et al., 2012). Therefore, it is imperative to use plant tissue culture method for large scale production of clonal planting material of the species from superior genotypes for quick rejuvenation. Lack of knowledge of genetic background is the main disadvantage associated with seedling material. Whereas, excessive leaching of phenolics, endogenous contamination and poor multiplication are the problems associated with the explants from mature trees (Beena et al., 2012). Though adult tissue is associated with problems, micropropagation through axillary shoot proliferation is considered safer and preferred for commercial propagation of timber woods as it maintains the genetic stability compared to organogenesis (McCown and McCown, 1987). Only two reports are available on micropropagation of *Melia dubia* with very poor multiplication rate and using nodal explants of 3-6 weeks-old in vitro raised seedlings (Chinnaraj et al., 2011; Ram et al., 2012). However, in vitro propagation through axillary shoot proliferation using nodal segments from mature trees has been reported for many other tree species of the family Meliaceae namely, *Azadirachta indica* (Venkateswarlu et al., 1998; Chaturvedi et al., 2004; Arora et al., 2010), *Azadirachta excelsa* (Liew et al., 2000), *Melia azedarach* (Thakur et al., 1998; Husain and Anis, 2009), *Toona ciliata* (Mroginski et al., 2003), *Khaya senegalensis* (Hung and Trueman, 2011), *Khaya ivorensis* (Mathias, 1988), *Swietenia macrophylla* (Lee and Rao, 1988) and *Cedrela odorata* (Maruyama et al., 1989). For large scale production, efficiency of propagation method is important, at the same time genetic stability also has prime concern. It has been extensively reported that tissue culture- induced genetic variations (somaclonal variation) are quite common in clonally propagated plants (Larkin and Scowcroft, 1981). Therefore, it is of immense significant to assure the genetic fidelity of in vitro raised plants at an early stage. Various markers are available to evaluate the genetic stability of the plants. Random amplified Polymorphic DNA (RAPD) is one of the cheapest methods among them (Rout and Das, 2002). As such there is no report on efficient protocol for mass clonal propagation from the superior genotypes and assessment of genetic stability of *M. dubia.* Therefore, the present work was aimed to develop micro propagation protocol for rapid and large scale production of clonal planting material of superior genotypes through axillary shoot proliferation as well as assessment of genetic stability using RAPD markers.

Materials and Methods

Explants collection, processing and surface sterilization

Experiments were carried out in plant tissue culture laboratory of the Institute of Wood Science and Technology (IWST), Bangalore during 2008 to 2011. Newly grown shoot segments, were collected from the plus trees selected from field station of Central

Silvicultural Zone, Karnataka. After excision of leaves, single nodal segments of 2.5 - 3.0 cm long explants were prepared for surface sterilization. To remove dust particles and reduce fungal contamination, explants were dipped in 0.1% (*v/v*) Polyoxyethylene sorbitan monooleate (Tween - 80; Himedia, India) liquid solution for 15 minutes and 0.1% (*w/v*) solution of Bavistin (Carbendazim 50% WP- a systemic fungicide) for 10 min with 5 - 6 through washes in double-distilled water after each treatment. Surface sterilization of the explants was carried out with 70 % (*v/v*) ethanol for 50 seconds followed by 0.1% (*w/v*) $HgCl_2$ solution for 8 - 10 min. Explants were washed 5 - 6 times with sterile distilled water after each treatment to remove the traces of sterilants. Cut ends of the surface sterilized explants were trimmed aseptically to minimize leaching and inoculated vertically on shoot initiation medium.

Culture medium and growth conditions

Based on the results of preliminary experiments on type of media and additives, for all shoot initiation and shoot multiplication experiments, Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) containing 3% (*w/v*) sucrose, additives (ascorbic acid - 284 µM, citric acid - 130 µM, L - cysteine - 206 µM, Adenine sulphate -7.23 µM and Gibberellic acid (GA_3) - 13.57 µM), agar (Hi-media) 0.7% (*w/v*) were used along with auxins and cytokinis. pH of the medium was adjusted to 6.4 (before autoclaving) and 20 ml of medium was dispensed into each 150 × 25 mm Borosil culture tubes and culture tubes were plugged with non-absorbent cotton wrapped in cheese cloth for shoot induction. For in vitro rooting, ½ MS (major inorganic salts reduced to half strength) medium was used. For shoot multiplication and in vitro rooting, 40 ml of medium was dispensed into 10 × 6 cm (250 ml) glass bottles. Dispensed media were autoclaved at 1.06 kg cm^{-2} at 121° C temperature for 20 min. The cultures were incubated in a growth room at 25 ± 2° C containing fluorescent lamps (Philips, Kolkata, India) providing 27 µE m^{-2} s^{-1} of light.

Shoot induction

Two separate experiments were carried out for shoot initiation. In the first experiment, various concentrations (0.44, 1.33, 2.22, 4.44, 6.66, 8.88 and 11.10 µM) of BAP and Kinetin (Kn) were tested for maximum bud break. In the second experiment, BAP (2.22 µM) alone was tested in combination with auxins; NAA and indole-3 acetic acid (IAA) at different concentrations (0.27, 0.54 and1.10 µM) for high frequency bud break and their subsequent growth. Data was recorded after 4 weeks.

Shoot multiplication

For shoot multiplication, clumps of 2 - 3 shoots were taken and inoculated in shoot multiplication media containing various concentrations of cytokinins; BAP and Kinetin(0.44 µM- 8.88 µM), TDZ (1.14 & 2.27 µM) alone or in combination with NAA and IAA (0.27 & 0.54 µM). Data was recorded after four weeks for shoot number, shoot length and quality of shoot.

In vitro rooting and acclimatization

Tissue culture raised microshoots of 3 - 4 cm in length with 2 - 3 nodes were tested with various concentrations (0.49, 1.50, 2.47, 4.93 and 7.40 µM) of IBA, IAA and NAA (0.49, 1.50, 2.47 and 4.93 µM) either alone or in combinations for in vitro rooting. The rooted plantlets were washed to remove agar, transferred to plastic cup containing

potting mixture of vermiculite, sand and soil (1 : 2 : 1 *v/v/v*) and placed under a polyglobule for 4 weeks in mist chamber at 30 ± 5ºC temperature and > 90% relative humidity (RH). Later on the plants were kept in mist chamber without cover for 4 weeks and then shifted to a shade net (50% shade) for another 2 weeks before placing it in open nursery. After successful acclimatization plants were evaluated for growth performance at an interval of two months up to ten months.

Genetic stability analysis of the micro propagated plants

DNA extraction and PCR amplification

Genomic DNA was extracted from fresh, young leaf tissue derived from ten randomly selected in vitro raised plants through axillary shoot proliferation and mother plants, following the cetyl trimethyl ammonium bromide (CTAB) method according to Dhillon and co-workers (2007). The quantity of DNA was measured by using nanometer (Thermo scientific-Nanodrop, 1000, 3.70).

Out of 20 decamer oligonucleotide primers from operon technologies Inc. (Operon, Alameda, California) tested, 11 decamer primers [series A, C and Q OPA-05 (AGGGGTCTTG), 09 (GGGTAACGCC), 10 (GTGATCGCAG), 11 (CAATCGCCGT), 13 (CAGCACCCAC), 16 (AGCCAGCGAA); OPC - 14 (TGCGTGCTTG); OPQ - 13 (GGAGTGGACA), 15 (GGGTAACGTG), 16 (AGTGCAGCCA), 17 (GAAGCCCTTG)] were selected for Polymerase chain reaction (PCR) amplification. PCR was performed in accordance with the protocol of Williams et al. (1990). Amplification was carried out in a 25 µl reaction volume containing 30 ng of genomic DNA as template, 2.5 µl of 10X PCR buffer, 1.5 mM $MgCl_2$, 10 pmol of primer, 2.0 µl of 0.8 mM dNTP mix (Bangalore, Genei) and 1.5 units of Taq (*Thermus aquaticus*) DNA polymerase (Bangalore, Genei). The amplification reaction was carried out in a Mastercycler gradient (Eppendorf, Germany) with cyclic profiles: initial denaturation at 94^o C for 3 minutes, followed by 44 cycles each, consisting of denaturation at 94^o C for 45 s, annealing at 35^o C for 45 s and extension at 72^o C for 1 min. The final extension cycle was for 5 min at 72^oC temperature. The amplification products were separated based on size by gel electrophoresis in 2.0% agarose gel in 1X TAE buffer and stained in ethidium bromide (Bangalore, Genei) solution and gel was observed in gel documentation system (Herolab, Germany). DNA fingerprinting with RAPD markers were performed thrice and only reproducible bands in the range of 300 to 3000 bp were scored.

Experimental design and statistical analysis

For shoot initiation experiments, 10 explants were taken for each treatment. Four clumps of shoots (3 - 4 shoots in each clump) were taken in each bottle and five such bottles were taken per treatment for shoot multiplication and in vitro rooting. Each experiment was repeated thrice. Experiments were set up according to a completely randomized design. Variance (one way or single factor analysis) in treatment means and standard errors were determined, followed by the least significant difference (LSD) test at $P \leq 0.05$ to compare means. DNA fingerprinting with RAPD markers was performed three times and only quality reproducible bands in the range of 350-2500 bp were scored. DNA banding pattern generated by RAPDs was recorded

as 1 for presence of band and 0 for its absence. Genetic difference between the parents and their progeny was calculated according to the RAPD marker data set as described by Ward.

Results and Discussion

Among the two cytokinins tested, BAP (2.22 µM) proved to be best in terms of percentage of bud break and shoot length **(**Table 1). However, combined use of BAP with auxins (NAA and IAA) further enhanced the percentage of bud break as compared to BAP alone in the medium. Maximum (90%) bud break with an average shoot length of 2.8 cm was achieved by combination of 2.22 µM BAP and 0.54 µM NAA within 4 weeks period (Table 2, Fig 1A). Combined use of BAP and NAA was also proved to be most effective for shoot initiation in *Aegle marmelos* and *Tectona grandis* (Shirin et al., 2005; Nayak et al., 2007).

Concentrations of BAP above 2.22 µM were inversely proportional to shoot number and shoot length which produced dwarf shoots. Increase in the concentrations of BAP also had negative effect on shoot initiation and its subsequent growth in *Bambusa pallida* (Beena and Rathore, 2012). Addition of auxins in the multiplication medium posed problem of callusing at the shoot base, which in turn hindered shoot growth. It may be due to the cells that have attained juvenility through biochemical changes under culture conditions, required no exogenous auxins. Therefore medium supplemented with BAP (2.22 µM) alone was found to be ideal for shoot multiplication which produced 4.92 fold shoots within 4 weeks period (Table 3, Fig 1B&C). Similarly, shoot multiplication of *Helianthus annuus* was optimal from half shoot apices cultured on MS media with 0.44 – 4.44 µM BAP, whereas auxins promoted callusing and inhibited shoot multiplication (Paterson, 1984).

Among the various auxins (IAA, IBA and NAA) used for in vitro rooting, 2.47 µM IBA favored the highest (98.00%) rooting, root number (4.33) and root length of 4.41cm (Table 4 and Fig. 1D&E). But at higher concentration (4.93 µM), it produced numerous (8.97) small roots at the base of shoots with callus formation. These shoots dried within 3-4 weeks period. Whereas, lower concentration of IBA resulted in delayed rooting and roots were found weak and thin. Generally, necessity of an auxin and reduced salt of MS medium has been found to be better than full MS medium in many woody species (Annapurna and Rathore, 2010). The present study is in accordance with the results obtained in *Melia azedarach and Azadirachta indica* where, superiority of IBA over the other auxins have been proved (Sen et al., 2010; Arora et al., 2010; Arora et al., 2011). Contrary to this, Joshi and Thengane (1996) reported IAA to be best auxin for in vitro rooting in *Azadirachta indica*. Survival rate of micro propagated plants after transplantation was 90% (Fig 1F&G). Hardened plants attained height of 109.48 cm and collar diameter of 40.07 mm in ten months period in an open nursery (Fig. 1H). Successful hardening with 67.6% and 95% was also achieved from woody species *Garcinia indica* and *Bambusa pallida* (Meera and Manjushri, 2005; Beena et al., 2012). RAPD has been used as a useful tool for investigating the genetic fidelity of tissue cultured plants by several researchers (Olmos et al., 2002; Gupta et al., 2009).

Table.1 Effect of cytokinins (BAP or Kn) in MS medium on shoot initiation from nodal shoot segment of *M. dubia* after 4 weeks

Cytokinins (µM)		Nodal shoot segment		
BAP	Kn	% of bud break	No. of Shoots/explant	Average shoot length (cm)
0.00	0.00	NR	1	NR
0.44	-	59.47^{e}	1	0.51^{c}
1.33	-	67.60^{d}	1	1.00^{b}
2.22	-	**85.03^{a}**	1	**1.97^{a}**
4.44	-	77.21^{b}	1	1.65^{b}
6.66	-	70.03^{c}	1	1.37^{b}
8.88	-	55.30^{f}	1	0.95^{b}
11.1	-	25.13^{j}	1	0.60^{c}
-	0.44	14.19^{l}	1	0.13^{d}
-	1.33	25.80^{j}	1	0.60^{c}
-	2.22	40.54^{g}	1	1.42^{b}
-	4.44	35.09^{h}	1	1.24^{b}
-	6.66	32.17^{i}	1	1.03^{b}
-	8.88	26.08^{j}	1	0.47^{c}
-	11.1	19.79^{k}	1	0.45^{c}
CD		**0.97**	**-**	**0.27**

Treatments followed by different letters are significantly different from each other. CD, Critical difference at $p \leq 0.05$

Table.2 Effect of auxins (NAA and IAA) in MS medium with 2.22 µM BAP on shoot initiation from nodal shoot segment of *M. dubia*

Cytokinin (µM)	Auxins (µM)		% of bud break	No. of shoots per explants	Average shoot length (cm)
BAP	NAA	IAA			
2.22	0.27	-	85.42^{c}	1	1.43^{c}
2.22	0.54	-	90.00^{a}	1	2.80^{a}
2.22	1.10	-	82.42^{d}	1	1.10^{c}
2.22	-	0.27	60.12^{f}	1	1.00^{c}
2.22	-	0.54	87.34^{b}	1	2.11^{b}
2.22	-	1.10	75.43^{e}	1	1.20^{c}
CD			**0.60**	**-**	**0.63**

Treatments followed by different letters are significantly different from each other. CD, Critical difference at $p \leq 0.05$

Table.3 Effect of PGRs (auxins and cytokinins) in MS medium supplemented with additives on shoot multiplication from shoot clump (2-3 shoots) of *M. dubia*

PGRs (µM)					Shoots per clump	Shoot length (cm)	Callus intensity
BAP	KN	NAA	IAA	TDZ			
-	-	-	-	-	**3.00^{k}**	1.13^{d}	+
0.44	-	-	-	-	7.83^{b}	1.97^{c}	+
2.22	-	-	-	-	**9.83^{a}**	**3.73^{a}**	**+**
4.44	-	-	-	-	7.36^{c}	2.57^{b}	++
8.88	-	-	-	-	5.24^{g}	1.50^{d}	+++
-	0.44	-	-	-	3.20^{k}	1.90^{c}	+
-	**2.22**	-	-	-	3.67^{j}	2.67^{b}	++
-	4.44	-	-	-	3.17^{k}	2.13^{c}	++
-	8.88	-	-	-	3.07^{k}	1.87^{c}	+++
2.22	-	0.27	-	-	7.00^{d}	1.40^{d}	+++
2.22	-	0.54	-	-	4.33^{i}	1.43^{d}	++++
2.22	-	-	0.27	-	6.56^{e}	1.59^{d}	+++
2.22	-	-	0.54	-	5.30^{g}	1.56^{d}	++++
-	2.22	0.54	-	-	5.27^{g}	1.07^{d}	++
-	2.22		0.54	-	6.33^{f}	1.30^{d}	++
-	-	-	-	1.14	5.33^{g}	1.07^{d}	+++
-	-	-	-	2.27	4.97^{h}	**0.80^{e}**	**++++**
CD					**0.16**	**0.10**	

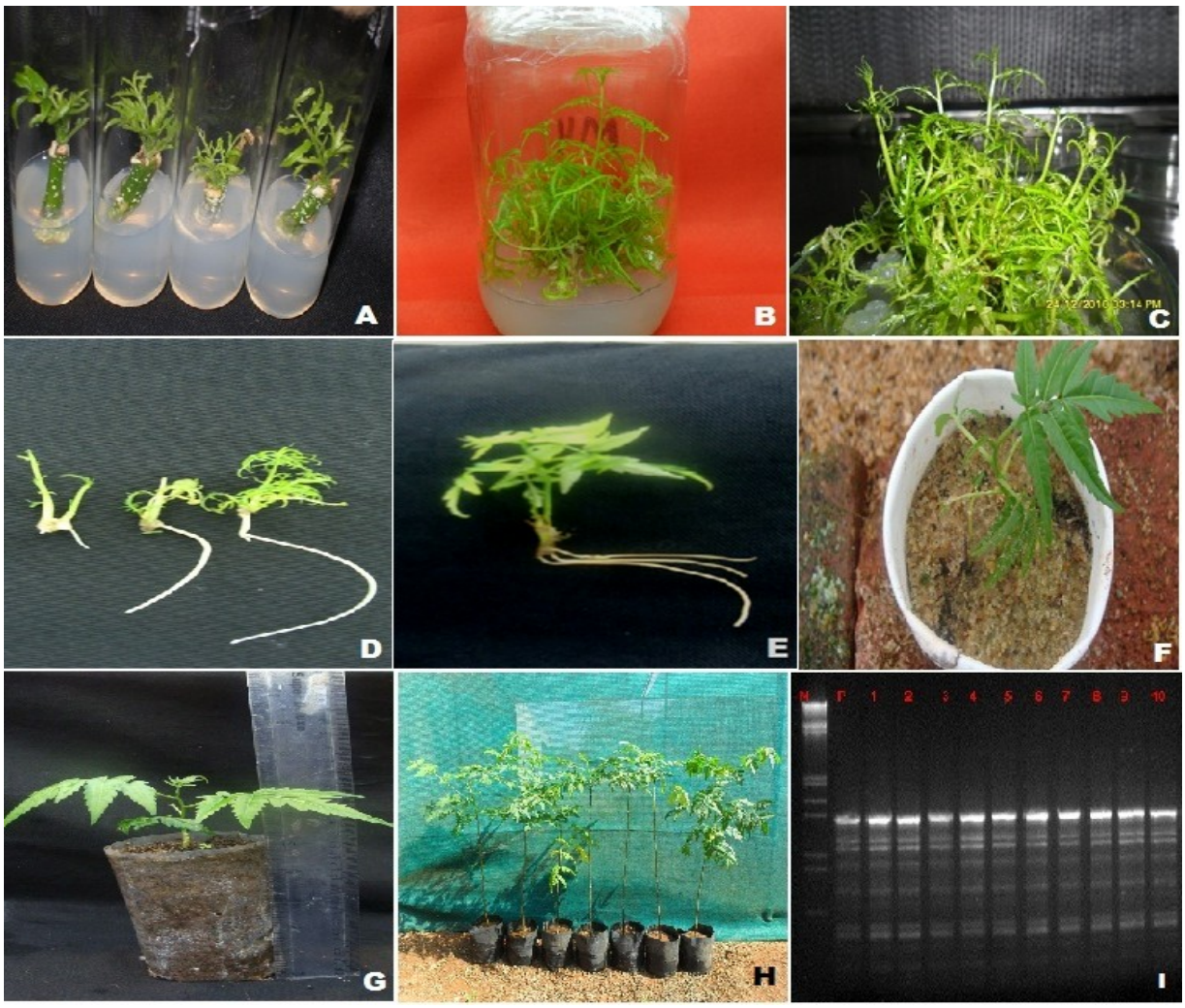
Treatments followed by different letters are significantly different from each other. CD, Critical difference at $p \leq 0.05$
+Without callus, ++ minute callus, +++ Moderate callus and ++++ vigorous callus growth.

Table.4 Effect of various concentrations of auxins (IBA, IAA and NAA) on in vitro rooting from shoots of *M. dubia* on ½ MS medium.

Auxins (µM)			% of Root induction	No. of roots/clump	Root length (cm)
IBA	IAA	NAA			
-	-	-	NR	NR	NR
0.49	-	-	90.00^{d}	1.27^{g}	1.37^{e}
1.50	-	-	95.00^{b}	3.22^{d}	3.23^{b}
2.47	-	-	98.00^{a}	4.33^{b}	4.41^{a}
4.93	-	-	93.41^{c}	8.97^{a}	1.77^{d}
7.40	-	-	80.53^{e}	4.0^{c}	1.50^{e}
-	0.49	-	35.10^{j}	1.33^{g}	0.80^{g}
-	1.50	-	61.00^{g}	1.63^{f}	1.27^{e}
-	2.47	-	70.00^{f}	2.63^{e}	2.30^{c}
-	4.93	-	49.00^{h}	1.17^{g}	1.13^{f}
-	7.40	-	29.00^{k}	1.37^{g}	0.77^{g}
-	-	0.49	11.00^{n}	1.40^{g}	0.47^{h}
-	-	1.50	NR	NR	NR
-	-	2.47	NR	NR	NR
-	-	4.93	NR	NR	NR
0.49	0.49	-	45.00^{i}	2.50^{e}	1.03^{f}
0.49	-	0.49	22.21^{m}	1.17^{g}	0.53^{h}
CD			**1.14**	**0.19**	**0.13**

Treatments followed by different letters are significantly different from each other. CD, Critical difference at $p \leq 0.05$. NR, Not Responded

Figure.1 In vitro propagation of *M. dubia* through axillary shoot proliferation and evaluation of genetic fidelity



A: In vitro shoot initiation of *M. dubia* in MS medium containing 2.22 µM BAP and 0.54 µM NAA with additives.
B&C: In vitro shoot multiplication of *M. dubia* in MS medium containing 2.22 µM BAP with additives.
D&E: In vitro rooting of *M. dubia* in ½ MS media containing 2.47 µM IBA.
F&G: Hardened plants of *M. dubia* after 2 months following acclimatization.
H: Hardened plants of *M. dubia* after 10 months
I: Amplified bands obtained in primer OPA-09, M: 100bp Ladder, P: parent plant and 1-10: micropropagated plants raised from one year old shoot multiplication cultures

Table.5 Details of RAPD analysis of in vitro regenerated plantlets using 11 decamer primers

Primers	Sequence (5'- 3')	No. of bands amplified	M.W.
OPA-05	AGGGGTCTTG	5	3099
OPA-09	GGGTAACGGG	11	3053
OPA-10	GTGATCGCAG	7	3068
OPA-11	CAATCGCCGT	6	2988
OPA-13	CAGCACCCAC	5	2942
OPA-16	AGCCAGCGAA	4	3046
OPC-14	TGCGTGCTTG	6	2000
OPQ-13	GGAGTGGACA	6	2850
OPQ-15	GGGTAACGTG	3	4100
OPQ-16	AGTGCAGCCA	10	3000
OPQ-17	GAAGCCCTTG	9	3500

In the present study, out of the eleven decamer oligonucleotides tested for genetic fidelity by RAPD, maximum number (11) of bands were obtained in OPA-09 (Table 5 and Fig 1I), whereas minimum (3) numbers of band were obtained in OPQ-15. Total 72 bands were produced with an average percentage of 6.55 bands per primer. The bands obtained using all 11 primers were found to be monomorphic across all the micropropagated plants confirming the genetic stability of regenerated plants. Stability of plants raised through axillary shoot proliferation has also been reported in *Robinia pseudoacacia* and *Mucuna pruriens (*Shu et al., 2003; Sathyanarayana et al., 2008).

In conclusion, the protocol developed for micropropagation of *Melia dubia* (Cavanilles) from explants of mature trees is safe for large scale production of clonal plants for operational planting. Present investigation also helps to conserve the species and forms the basis for further research.

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