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Meta-topolin Improves In Vitro Morphogenesis, Rhizogenesis and Biochemical Analysis in *Pterocarpus marsupium* Roxb.: A Potential Drug-Yielding Tree

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Abstract

Meta-topolin (*m*T), a benzyladenine analog [*N* 6-(3-hydroxybenzylamino) purine] is a highly active cytokinin. The present study evaluates the efficiency of two aromatic cytokinins, *m*T and BA for inducing in vitro regeneration in a woody legume *Pterocarpus marsupium* (Roxb.) using cotyledonary node (CN) explants. Of the two cytokinins tested, *m*T-derived cultures resulted in better shoot multiplication and rhizogenesis than BA. Among the different doses of *m*T, maximum shoot (9.58 ± 0.30) induction per explant and average shoot length (4.12 ± 0.05 cm) were recorded on Murashige and Skoog (Physiol Plant 15:473–497, 1962) medium containing 7.5 µM *m*T, after 6 weeks of culture. The combined effect of cytokinin and auxin was tested, auxin was mixed with optimum doses of BA or *m*T separately and the effect of combination was studied. Among the cytokinin–auxin combinations, the highest number of shoots (17.44 ± 0.25) per explant and average shoot length (5.72 ± 0.18 cm) were achieved on MS medium containing 7.5 µM *m*T with 1.0 µM αnaphthalene acetic acid in 85% of the cultures after 12 weeks. *Meta*-topolin alone or in combination with auxin has shown an increase in the quality and number of shoots in comparison to BA. In vitro rhizogenesis in individually regenerated microshoots was carried out on half-strength MS medium augmented with 1.0 µM indole-3-butyric acid via a two-step procedure method. After 4 weeks, 7.35 ± 0.11 roots per shootlet with an average root length of 4.54 ± 0.10 cm were recorded in *m*T-derived microshoots. The well-developed plantlets were acclimatized in a separate batch of single CN explant-derived plantlets. About 80% survival rate was recorded for *m*T-derived plantlets. Biomass and photosynthetic pigments were also improved in *m*T-derived plantlets, when compared with the BA derived. Analysis of genetic homogeneity of ten micropropagated plantlets was done through RAPD. Out of 40 RAPD primers, 29 primers produced clearly scorable monomorphic bands, thus exhibiting complete genetic uniformity among in vitro regenerated plantlets.

Keywords Acclimatization · Cotyledonary node · Fabaceae · Genetic fidelity · Regeneration · Woody legume

Introduction

Many countries, including India, are facing substantial problems in maintaining forest tree resources because of their uncontrolled exploitation and climate change. *Pterocarpus marsupium* Roxb. (Fabaceae), commonly known as "Bijasal" or "Indian Kino" is a potential herbal drug-yielding forest tree legume of India. In the Indian system of medicine, various parts of this plant have been used to cure diabetes, elephantiasis, leprosy, diarrhoea, chronic ulcers and dysentery, since ancient times (Maurya et al. 2004; Mohire et al. 2007). This tree species possesses several pharmacological properties such as anti-inflammatory, hypocholesterolemic, antimicrobial, antifungal, antianalgesic, hepatoprotective, cardiotonic, cyclooxygenase (COX-2) inhibition and anti-hypertriglyceridaemic (Manickam et al. 1997; Hougee et al. 2005). Heartwood extracts contain a potent antioxidant (5,7,2–4 tetrahydroxy 6–6 isoflavone-glucoside) which is used to cure cardiovascular disease, vasodilation and inhibition of platelet aggregations (Mohire et al. 2007). Stem bark extract contains very important constituents having cancer-preventive properties such as lupeol, tetradecanoic acid and octadecadieonic acid (Remsberg et al. 2008). The Global Forest Resources Assessment (2005) report placed this plant under the category

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of critically endangered plant species (Garzuglia 2006). Low seed-germination percentage and early loss of seed viability are acute problems for conventional propagation of the tree. In addition, overexploitation of the native natural stands for wood and medicine has resulted in a sharp decline of its natural habitat. The use of alternative propagation methods is vital for conservation of the species. Rapid in vitro regeneration through plant tissue culture techniques is very much in use for the propagation of such woody species (Javed et al. 2013; Gentile et al. 2014). A few successful reports on in vitro regeneration of *P. marsupium* have been documented using 6-benzyladenine (BA) (Chand and Singh 2004; Husain et al. 2007), however, the incidences of hyperhydricity, shoot tip necrosis, poor rooting and low survival rate limit the application of these protocols. Further, BA has many inherent drawbacks in in vitro propagation systems such as inhibiting rooting efficiency (Werbrouck et al. 1995), maintenance of histogenic stability (Bogaert et al. 2006) and physiological abnormality (Amoo et al. 2011). Therefore, it becomes crucial to find a substitute for BA that will maintain multiplication rate production. There are many reports showing improvement in mass propagation with the use of *meta*-topolin (*m*T), in place of 6-benzyladenine (BA) (Kubalakova and Strnad 1992; Strnad et al. 1997; Aremu et al. 2012). *Meta*-topolin differs from BA by the existence of a hydroxyl group in the aromatic side chain that facilitates the formation of a *O*-glycoside (Werbrouck et al. 1996), which is capable of rapid conversion to the active form of nucleosides, nucleotides or free bases when required (Strnad et al. 1997). These free bases of *m*T showed high activity in micropropagation systems in several plant species (Werbrouck et al. 1996). Use of *m*T in plant tissue culture has gained increasing interest due to reports on various important parameters such as multiple shoot induction (Bairu et al. 2007), improving physiological and biochemical traits (Aremu et al. 2012; Malá et al. 2013), successful rooting and easy acclimatization (Gentile et al. 2014).

Given this, micropropagation of *P. marsupium*, a woody legume was carried out using *m*T as a potential alternate of BA. This alternative hormone was able to induce a significant number of good quality shoots, better rooting efficiency during the in vitro phase and successful acclimatization in ex vitro conditions. The genetic fidelity of the regenerants was also evaluated using RAPD analysis of single CN explant-derived plantlets.

Materials and Methods

Seed Germination, Procurement of Explants, Nutrient Media and Culture Conditions

The fruits were collected from healthy trees of *P. marsupium* growing in the forest region of Rajahmundry (Andhra Pradesh, India) in the month of March. The seeds were scarified mechanically, disinfected and cultured in vitro using the reproducible method devised by Anis et al. (2005) for obtaining healthy axenic plantlets. Cotyledonary node explants (1.0–1.5 cm, without cotyledons, Fig. 1a) were excised from 20-day-old axenic plantlets and inoculated on MS medium (Murashige and Skoog 1962) supplemented with various plant growth regulators.

For multiple shoot bud induction, MS basal medium supplemented with 6-benzyladenine (BA) and *meta*-topolin (*m*T) at various concentrations (0.5, 1.0, 2.0, 2.5, 5.0, 7.5, 10.0 or 12.0 µM), was used individually or in different combination with auxins such as indole-3-acetic acid (IAA), α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) at various concentrations (0.25, 0.5, 1.0, 2.0, or 2.5 µM) in glass culture tubes (25 × 150 mm, Borosil, India) and Erlenmeyer flasks (100 ml, Borosil, India). Three percent (w/v) sucrose (Thermo Fisher Scientific, India) was added to the media as a sole carbon source. The medium was gelled with 0.8% (w/v) agar (Thermo Fisher Scientific, India), for in vitro culture growth. The pH of the media was adjusted to 5.8 using 1N NaOH or 1N HCl before autoclaving at 121 °C and 1.06 kg cm^{-2} pressure for 19 min.

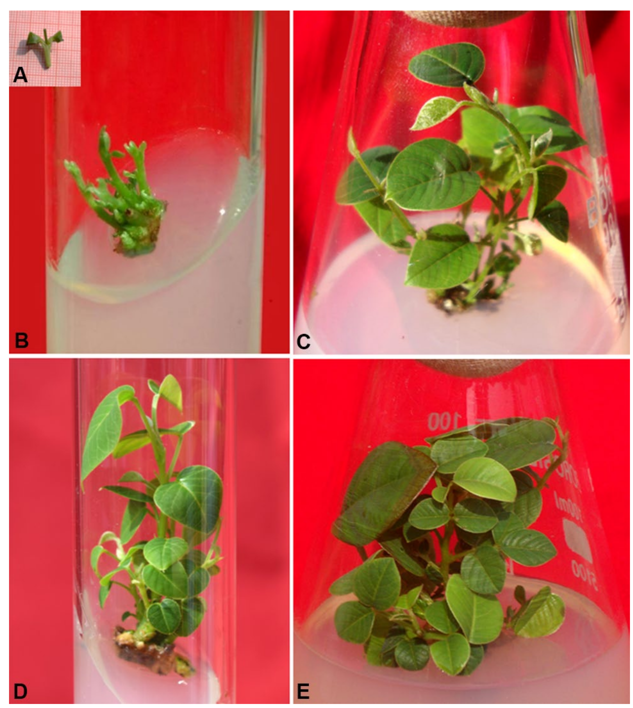

Fig. 1 Multiple shoot induction and proliferation. **a** CN explant obtained from 20-day-old axenic plantlet derived from seed. **b** Multiple shoots induction on MS + BA (7.5 µM), after 6 weeks of culture. **c** Multiple shoot proliferation on MS + BA (7.5 µM) + NAA (1.0 µM), after 12 weeks of culture. **d** Multiple shoot induction on MS + *m*T (7.5 µM), after 6 weeks of culture. **e** Multiple shoot proliferation on MS + *m*T (7.5 µM) + NAA (1.0 µM), after 12 weeks of culture

All culture vessels were incubated at 24 ± 2 °C, 16/8 h (day–night) photoperiod with PPFD of 50 µmol m^{-2} s^{-1} provided by white fluorescent tubes lights (40W; Philips, Electronics India Ltd., Kolkata India). Relative humidity was maintained at 50–60% and regularly checked by a thermohygrometer (Testo, India Pvt. Ltd.). All cultures were transferred to fresh medium every 2 weeks of incubation. The data on percent response, number of shoots and shoot length were recorded, every 3 weeks of culture.

Rooting and Acclimatization

A two-step method for in vitro root induction established by Anis et al. (2005) was followed. BA- or *m*T-derived, healthy and well elongated microshoots (≥ 4 cm) with more than three fully expanded leaves were isolated separately in two groups (BA- and *m*T-derived microshoots) from 12-week-old cultures and their basal end portions were pre-treated with various high doses of IBA (50, 100, 150 µM) in half-strength MS liquid medium with 2% (w/v) sucrose employing a filter paper bridge for various durations. Thereafter, pre-treated microshoots were transferred onto half-strength MS semisolid medium augmented with low doses of IBA (0.0, 0.1, 0.5, 1.0, 1.5 or 2.0 µM) and 2% (w/v) sucrose, solidified with 0.25% (w/v) Phytagel™ (Sigma-Aldrich, India). Microshoots with no pre-treatment failed to show rhizogenesis at any of the low doses of IBA. The data on percent of rooting, number of roots, mean root length were recorded after 4 weeks of culture transfer (Table 1).

Shootlets with well-developed roots were removed from the culture tube and gently washed with running tap water to remove any adherent phytagel. Plantlets were transplanted to 10-cm diameter thermocol cups containing sterile Soilrite™ (Kelteck Energies Ltd., Bangalore, India). The cups were covered with transparent polythene bags as a safeguard to ensure high humidity. These transplanted cups were placed under a 16/8 h (day/night) photoperiod cycle with artificial light (irradiance of 150 µmol m^{-2} s^{-1} PPFD) provided by white fluorescent tubes and watered with ¼ inorganic solution of MS medium every alternative day for 2 weeks. Thereafter, polythene bags were opened and gradually removed to acclimatize the plantlets to natural conditions. After 4 weeks, these well acclimatized plantlets were transferred to pots having normal garden soil maintained in a greenhouse under natural day light conditions.

Photosynthetic Pigment and Biomass Content Analysis

Chlorophyll and carotenoid pigments were extracted from a 3-month-old leaf tissue of BA- and *m*T-derived plantlets (grown under similar condition), following the Mackinney (1941) method. 200 mg of fresh leaves were ground in 80% acetone solution and filtered with filter paper (Whatman no. 1). The obtained extract was diluted to a final volume of 10 ml and quantification was performed by spectrophotometer (US-1700 Pharma Spec, Shimadzu, Kyoto, Japan). Optical density for the chlorophyll solution was read at 645 nm, 663 nm and for carotenoids at 480 nm and 510 nm wavelengths. These pigments were expressed as milligram (mg) per gram (g) of fresh weight (FW). Ten replicates were taken from each treatment (BA- or *m*T-derived plantlets) for the assessment of photosynthetic pigments and spectrophotometrical readings were repeated thrice.

Fresh weights of ten in vitro-raised plantlets (BA- and *m*T-derived) were recorded immediately, and then dried in

Table 1 Effect of IBA on root induction in pre-treated (100 µM IBA, for 5-days) microshoots, after 4 weeks of transfer

Pre-treated microshoots	IBA (µM)	% Response	No. of roots per microshoot Mean ± SE	Root length (cm) per microshoot Mean ± SE
BA derived	0.0	16	2.16 ± 0.06[g]	1.26 ± 0.03[h]
	0.1	23	3.14 ± 0.10[f]	2.02 ± 0.10[f]
	0.5	30	3.45 ± 0.07[e]	2.35 ± 0.09[e]
	1.0	50	4.53 ± 0.06[c]	3.65 ± 0.04[b]
	1.5	36	4.12 ± 0.11[d]	3.23 ± 0.07[c]
	2.0	20	3.65 ± 0.09[e]	3.04 ± 0.05[c]
*m*T derived	0.0	25	2.36 ± 0.07[g]	1.59 ± 0.04[g]
	0.1	35	3.54 ± 0.04[e]	2.25 ± 0.08[e]
	0.5	45	4.47 ± 0.15[c]	2.76 ± 0.12[d]
	1.0	75	7.35 ± 0.11[a]	4.54 ± 0.10[a]
	1.5	40	5.51 ± 0.06[b]	3.48 ± 0.10[b]
	2.0	30	5.28 ± 0.04[b]	2.82 ± 0.06[d]

Values represented means ± SE. Means followed by the same letter within column are not significantly different ($P = 0.05$) using Duncan's multiple range test shade for 24 h followed by drying in an oven maintained at 85 °C for 48 h. Dried plantlets were weighed to estimate the dry matter production and the mean values were expressed in milligram (mg) per plantlet (Table 2).

Genomic DNA Extraction and Genetic Fidelity Analysis

All the in vitro regenerated plantlets were maintained in a greenhouse under similar conditions as separate batches of single CN explant-derived plantlets. Each batch contained about 16–20 plantlets. Genomic DNA was extracted from a 3-month-old leaf tissue of 10 randomly selected plantlets from each batch, following the protocol established by Deshmukh et al. (2007). The quantitative analysis of extracted genomic DNA was tested using a Nanodrop spectrophotometer (Implen, Germany) and size clarity, reliability by 0.8% (w/v) agarose (Sigma-Aldrich, India) gel electrophoresis. Each DNA sample was diluted to 25 ng μl^{-1} in sterile double-distilled water.

Genetic homogeneity of regenerants was accomplished by the RAPD technique following the procedure of Williams et al. (1990). The preliminary screenings of the genomic DNA samples were carried out using 40 RAPD primers (Table 3). The PCR reaction for RAPD amplification was carried out on a Thermocycler (Biometra, T-Gradient, Germany). The PCR amplification mixture (25 µl) contained 10× buffer with $(NH_4)_2SO_4$, 25 mM $MgCl_2$, 10 mM dNTPs, 10 µM primers, 3 Unit Taq polymerase and 50 ng μl^{-1} template DNA. The PCR amplification temperature profile were used as the initial DNA denaturation step (94 °C for 5 min) followed by a 40 cycle program including a denaturation step (94 °C for 1 min), annealing step (35 °C for 1 min) and elongation step (72 °C for 2 min). Final extension was followed at 72 °C for 10 min. DNA-amplified products were separated by gel electrophoresis in 1.0% agarose gels. The DNA bands were visualized using a gel documentation system (Bio Rad, Hercules, CA, USA). The same banding patterns were considered to be homologous fragments, irrespective of intensity. Well-defined and reproducible bands were counted as present or absent for RAPD markers in each sample. The experiment was repeated three times to confirm reproducibility.

Statistical Analysis

All the experiments were repeated three times. Each treatment contained ten replicates. A single explant was cultured per culture test tube/flask. Data were examined using SPSS version 17 (SPSS Inc. Chicago, USA). Means were compared using Duncan's multiple range test (DMRT) at the 5% level of significance. All the results were expressed in means ± SE.

Results

Shoot Culture Initiation and Proliferation

The media without plant growth regulators (PGRs) were virtually unable to induce any response from the explants, whereas media enriched with increasing concentrations of the two cytokinins were able to promote shoot induction. The morphogenic response of CN explants (Fig. 1a) evaluated at different concentrations of BA or *m*T (0.5, 1.0, 2.0, 2.5, 5.0, 7.5, 10.0 or 12.0 µM) indicated an obvious effect of concentrations and type of cytokinin on multiple shoot induction. Of the various treatments, *m*T (7.5 µM) was found to be more effective than other concentrations with reference to multiple shoot induction. At this level, the maximum number of shoots (9.58 ± 0.30) per explant with an average shoot length (4.12 ± 0.05 cm) in 75% of the cultures was recorded, after 6 weeks of incubation (Table 4; Fig. 1d). On the other hand, the media enriched with BA (7.5 µM), could induce only a maximum number of 6.64 ± 0.17 shoots per explant with an average shoot length of 3.48 ± 0.24 cm with a 50% regeneration rate, after 6 weeks of incubation (Table 4; Fig. 1b). The concentration beyond the optimal level (7.5 µM) of both cytokinins showed no enhancement in shoot number, elongation or regeneration rate.

The synergistic effect of the optimal concentration of *m*T with auxins (NAA, IAA and IBA) at various concentrations was also evaluated for the enhancement in the multiplication

Table 2 Comparative analysis of survival rate and biomass content of 3-months-old in vitro-raised plantlets of *Pterocarpus marsupium*

Treatment	% Survival	sFW mg/plantlet	sDW mg/plantlet	rFW mg/plantlet	rDW mg/plantlet
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
BA derived	67.0 ± 2.55^b	348.0 ± 4.85^b	44.4 ± 2.31^b	079.2 ± 4.14^b	18.2 ± 0.86^b
*m*T derived	80.0 ± 3.53^a	401.6 ± 3.69^a	60.8 ± 2.39^a	115.0 ± 7.90^a	21.6 ± 1.03^a

Values are in mean ± SE. Means followed by the same letter within column are not significantly different ($P = 0.05$)

sFW shoot fresh weight, *sDW* shoot dry weight, *rFW* root fresh weight, *rDW* root dry weight

Table 3 Randomly amplified polymorphic DNA (RAPD) primers used to screen genetic fidelity of in vitro-raised plantlets of *Pterocarpus marsupium*

Sr. no.	Primers	Sequence (5′–3′)	No. of bands
1	OPA01	CAGGCCCTTC	5
2	OPA02	TGCCGAGCTG	0
3	OPA03	AGTCAGCCAC	4
4	OPA04	AATCGGGCTG	4
5	OPA05	AGGGGTCTTG	3
6	OPA06	GGTCCCTGAC	0
7	OPA07	GAAACGGGTG	3
8	OPA08	GTGACGTAGG	6
9	OPA09	GGGTAACGCC	5
10	OPA10	GTGATCGCAG	6
11	OPA11	CAATCGCCGT	4
12	OPA12	TCGGCGATAG	5
13	OPA13	CAGCACCCAC	3
14	OPA14	TCTGTGCTGG	0
15	OPA15	TTCCGAACCC	4
16	OPA16	AGCCAGCGAA	4
17	OPA17	GACCGCTTGT	5
18	OPA18	AGGTGACCGT	3
19	OPA19	CAAACGTCGG	3
20	OPA20	GTTGCGATCC	0
21	OPC01	TTCGAGCCAG	3
22	OPC02	GTGAGGCGTC	5
23	OPC03	GGGGGTCTTT	4
24	OPC04	CCGCATCTAC	0
25	OPC05	GATGACCGCC	3
26	OPC06	GAACGGACTC	0
27	OPC07	GTCCCGACGA	3
28	OPC08	TGGACCGGTG	0
29	OPC09	CTCACCGTCC	0
30	OPC10	TGTCTGGGTG	4
31	OPC11	AAAGCTGCGG	3
32	OPC12	TGTCATCCCC	0
33	OPC13	AAGCCTCGTC	3
34	OPC14	TGCGTGCTTG	7
35	OPC15	GACGGATCAG	6
36	OPC16	CACACTCCAG	0
37	OPC17	TTCCCCCCAG	3
38	OPC18	TGAGTGGGTG	0
39	OPC19	GTTGCCAGCC	3
40	OPC20	ACTTCGCCAC	3
Total			117
Average bands per primer			4.03

and elongation of the shoots. Moreover, overall mean values observed indicated that the explants respond better when the medium was enriched with different concentrations of NAA (Table 5). Indeed, MS medium containing NAA (1.0 µM) in combination with *m*T (7.5 µM) enhanced the multiplication rate in explants and evaluated as 17.44 ± 0.25 shoots per explant with an average shoot length (5.72 ± 0.18 cm) in 85% of the cultures, after 12 weeks of incubation (Table 5; Fig. 1e). However, an equimolar dose of BA and NAA, induced less shoots (13.22 ± 0.39) per explant and average shoot length (4.52 ± 0.18 cm) with 65% regeneration frequency, after 12 weeks (Table 5; Fig. 1c). Moreover, *m*T alone or in combination with auxin was more effective than BA in the induction of multiple shoots and proliferation from CN explants. In some cultures, callus was also observed at the base of the culture which was removed during subculturing as a precaution, as callus formation stunted the shoot multiplication rate.

Table 4 Effect of cytokinins on multiple shoot induction from CN explants, after 6 weeks of incubation

Cytokinins (µM)		% Response	No. of shoots per explant	Shoot length (cm) per explant
BA	*m*T		Mean ± SE	Mean ± SE
0.0		00	0.00 ± 0.00^{l}	0.00 ± 0.00^{h}
0.5		10	1.52 ± 0.17^{k}	1.58 ± 0.26^{g}
1.0		15	2.00 ± 0.26^{jk}	1.84 ± 0.16^{fg}
2.0		15	2.20 ± 0.14^{ij}	2.36 ± 0.05^{def}
2.5		20	3.86 ± 0.28^{fg}	2.58 ± 0.24^{cde}
5.0		30	4.28 ± 0.25^{ef}	3.00 ± 0.23^{bcd}
7.5		50	6.64 ± 0.17^{c}	3.48 ± 0.24^{cdef}
10.0		35	3.58 ± 0.16^{gh}	3.14 ± 0.25^{bc}
12.0		10	1.56 ± 0.07^{k}	2.23 ± 0.14^{efg}
	0.5	15	2.58 ± 0.09^{i}	1.82 ± 0.16^{fg}
	1.0	20	3.36 ± 0.21^{gh}	2.48 ± 0.17^{ef}
	2.0	25	4.54 ± 0.11^{e}	2.82 ± 0.26^{bcde}
	2.5	25	5.95 ± 0.18^{d}	3.00 ± 0.33^{bcde}
	5.0	35	7.86 ± 0.17^{b}	3.46 ± 0.28^{b}
	7.5	75	9.58 ± 0.30^{a}	4.12 ± 0.05^{a}
	10.0	25	6.59 ± 0.11^{c}	3.00 ± 0.10^{bcd}
	12.0	20	3.28 ± 0.06^{h}	2.34 ± 0.26^{def}

Values represented means ± SE. Means followed by the same letter within column are not significantly different ($P = 0.05$) using Duncan's multiple range test

Rhizogenesis and Acclimatization

No rooting was observed in non-pre-treated microshoots when transferred to PGR-free basal medium. However, basal ends of *m*T-derived microshoots, pre-treated in IBA solution at 100 µM for 5 days (Fig. 2a), following transfer onto ½ MS semisolid medium augmented with IBA at lower concentrations showed significant root numbers per microshoot, after 4 weeks of transfer. A significant increase in number of roots (7.35 ± 0.11) and their length

Table 5 Synergistic effect of various concentrations of auxins with optimal dose of two cytokinins on multiple shoot proliferation, after 12 weeks of incubation

Optimum cytokinin	Auxin (μM)			% Response	No. of shoots per explant	Shoot length (cm) per explant
	IAA	NAA	IBA		Mean ± SE	Mean ± SE
BA (7.5 μM)	0.25			65	8.52 ± 0.29[jk]	4.00 ± 0.10[defgh]
	0.50			65	9.35 ± 0.20[hi]	4.18 ± 0.07[def]
	1.00			65	11.83 ± 0.19[f]	4.22 ± 0.23[def]
	2.00			55	8.94 ± 0.16[ij]	3.86 ± 0.05[efgh]
	2.50			55	7.48 ± 0.20[l]	3.58 ± 0.23[gh]
		0.25		45	9.31 ± 0.16[hi]	4.10 ± 0.29[defg]
		0.50		45	10.43 ± 0.28[g]	4.34 ± 0.10[bcde]
		1.00		65	13.22 ± 0.39[d]	4.52 ± 0.18[bcd]
		2.00		60	9.65 ± 0.22[h]	4.26 ± 0.17[cdef]
		2.50		55	8.14 ± 0.16[k]	3.82 ± 0.08[efgh]
			0.25	30	7.10 ± 0.26[lm]	3.60 ± 0.14[gh]
			0.50	50	7.28 ± 0.24[lm]	3.86 ± 0.07[efgh]
			1.00	60	7.40 ± 0.26[lm]	4.14 ± 0.28[defg]
			2.00	40	6.95 ± 0.25[lm]	3.72 ± 0.16[fgh]
			2.50	45	6.74 ± 0.14[m]	3.46 ± 0.23[h]
*m*T (7.5 μM)	0.25			70	11.68 ± 0.18[f]	4.29 ± 0.25[cdef]
	0.50			70	12.48 ± 0.25[e]	4.48 ± 0.26[bcd]
	1.00			75	13.73 ± 0.14[d]	4.88 ± 0.17[b]
	2.00			65	11.72 ± 0.25[f]	4.36 ± 0.03[bcde]
	2.50			60	11.32 ± 0.21[f]	3.86 ± 0.21[efgh]
		0.25		70	13.86 ± 0.18[d]	4.38 ± 0.10[bcde]
		0.50		75	15.34 ± 0.18[b]	4.82 ± 0.24[bc]
		1.00		85	17.44 ± 0.25[a]	5.72 ± 0.18[a]
		2.00		70	14.62 ± 0.29[c]	4.50 ± 0.14[bcd]
		2.50		65	13.46 ± 0.13[d]	4.26 ± 0.23[cdef]
			0.25	60	9.75 ± 0.24[h]	4.10 ± 0.08[defg]
			0.50	55	10.42 ± 0.22[g]	4.26 ± 0.09[cdef]
			1.00	60	11.57 ± 0.14[f]	4.50 ± 0.10[bcd]
			2.00	65	9.92 ± 0.29[gh]	4.20 ± 0.08[def]
			2.50	60	9.68 ± 0.09[h]	3.74 ± 0.07[fgh]

Values represented means ± SE. Means followed by the same letter within the column are not significantly different ($P = 0.05$) using Duncan's multiple test

(4.54 ± 0.10 cm) per microshoot was attained in 75% of cultures on ½ MS medium augmented with 1.0 μM IBA, after 4 weeks of transfer (Table 1; Fig. 2d, e). Whereas, in the case of BA-derived microshoots, the maximum number of roots (4.53 ± 0.06) and lengths (3.65 ± 0.04) per microshoot were recorded in 50% of cultures in similar conditions and equimolar dose (Table 1; Fig. 2b, c). In vitro-raised healthy regenerants were taken out from the culture tube, after 4 weeks of root formation (Fig. 2c, e). These regenerants were acclimatized in autoclaved Soilrite™ (Fig. 2f), followed by their transfer to pots containing normal garden soil. Efficiency of in vitro rooting of *m*T-derived microshoots was high as compared to BA (Fig. 2c, e).

Photosynthetic Pigment, Biomass Content and Survival Rate

Comparative analysis of the photosynthetic pigments chlorophyll (*Chl* 'a' and *Chl* 'b'), and carotenoids (*Car*) was carried out in BA- and *m*T-derived plantlets. The *Chl* 'a', *Chl* 'b', and carotenoid contents of *m*T-derived plantlets showed a significantly higher concentration than BA-derived plantlets (Table 6). *m*T-derived plantlets showed *Chl* 'a' (1.50 ± 0.051), *Chl* 'b' (0.60 ± 0.033) and *Car* (0.53 ± 0.025) mg g^{-1} of FW, whereas *Chl* 'a' (1.02 ± 0.080), *Chl* 'b' (0.37 ± 0.034) and *Car* (0.34 ± 0.026) mg g^{-1} of FW was recorded in BA-derived plantlets (Table 6).

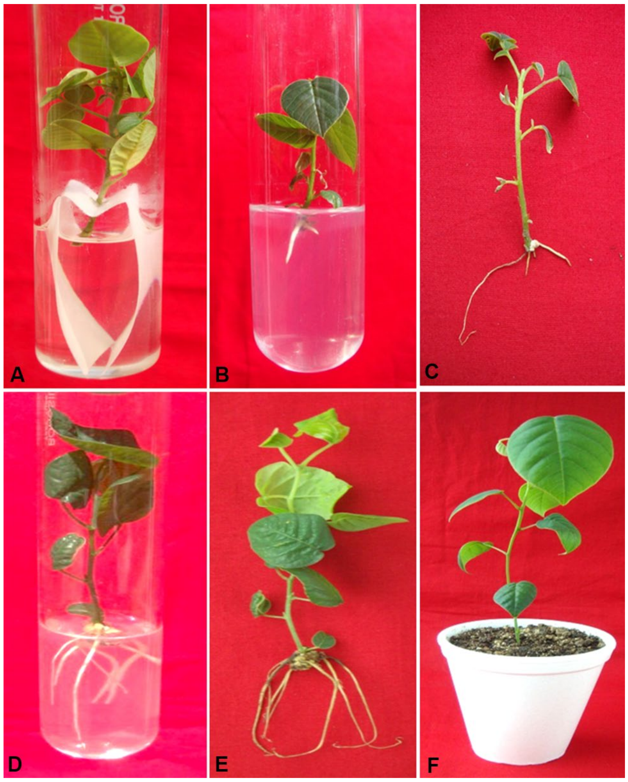

Fig. 2 Rooting and acclimatization. **a** Pre-treatment of microshoot on liquid medium, employing filter paper bridge method. **b** and **c** Root induction in BA-derived microshoot, after 3 and 4 weeks, respectively. **d** and **e** Root induction in *m*T-derived microshoot, after 3 and 4 weeks, respectively. **f** *m*T-derived plantlet, acclimatized in Soilrite

Biomass (fresh weight and dry weight) content was also better in *m*T-derived plantlets as compared to BA derived. 3-month-old in vitro-raised *m*T-derived plantlets showed mean values of biomass such as sFW (401.6 ± 3.69) mg per plantlets, sDW (60.8 ± 2.39) mg per plantlets, rFW (115.0 ± 7.90) mg per plantlets and rDW (21.6 ± 1.03) mg per plantlets (Table 2). More than 80% of *m*T-derived and 67% of BA-derived rooted plantlets survived, after 3 months of acclimatization (Table 2).

Assessment of Genetic Fidelity

Genetic fidelity analysis was carried out among the ten randomly selected in vitro regenerated plantlets, which were regenerated from single CN explants using RAPD markers. The markers were chosen because of ease in amplifying different regions of the genome and providing a broad range of discrepancy in regenerated plantlets at the molecular level as well as its cost effectiveness. A total of 40 RAPD primers were initially screened and finally 29 primers, produced clear, unambiguous and reproducible amplified bands (Table 3; Fig. 3). A total of 117 bands with an average of 4.03 bands per RAPD primer, among regenerants from single CN explants were scored and were found to be monomorphic in banding pattern (Table 3). No sign of polymorphism was obtained during the RAPD analysis among the single CN explant-derived plantlets. The RAPD marker banding profiles produced by OPA 10 and OPC 13 are shown in Fig. 3a and b, respectively.

Discussion

In vitro shoot regeneration and multiplication during micropropagation practices depend on the types of applied plant growth regulators, their concentrations, uptake, transport, metabolism and endogenous cytokinins levels of explants (Howell et al. 2003; Santner and Estelle 2009). Endogenous cytokinins are found in the form of free bases, ribosides, *N*-glucosides, *O*-glucosides and nucleotides in plants (Letham and Palni 1983). Similarly, N^6-sustituted purine derivative cytokinins are very important for regulation of physiological and organogenesis processes in plants (Letham and Palni 1983). The activity of cytokinins is also regulated by *N*-linked, *O*-linked glycosylation and conjugates of sugars and their derivatives (Drewes and van Staden 1989). Although, BA is widely used as one of the most efficient and reasonably priced cytokinins in plant tissue culture, it often induces disproportional growth or inhibition of rooting, toxicity and callus formation in a number of plant species (Magyar-Tábori et al. 2010; Gentile et al. 2014). These unwanted effects of BA may be due to its N^7- and N^9-glycosylation

Table 6 Comparative photosynthetic pigments concentration of 3-month-old leaf samples of in vitro-raised plantlets of *Pterocarpus marsupium*

Treatment	*Chl* 'a' mg g^{-1} of FW Mean ± SE	*Chl* 'b' mg g^{-1} of FW Mean ± SE	T*Chl* mg g^{-1} of FW Mean ± SE	*Car* mg g^{-1} of FW Mean ± SE
BA derived	1.02 ± 0.080^b	0.37 ± 0.034^b	1.40 ± 0.114^b	0.34 ± 0.026^b
*m*T derived	1.50 ± 0.051^a	0.60 ± 0.033^a	2.10 ± 0.084^a	0.53 ± 0.025^a

Values are in mean ± SE. Means followed by the same letter within column are not significantly different (P = 0.05)

Chl Chlorophyll, T*Chl* total chlorophyll, *Car* carotenoids

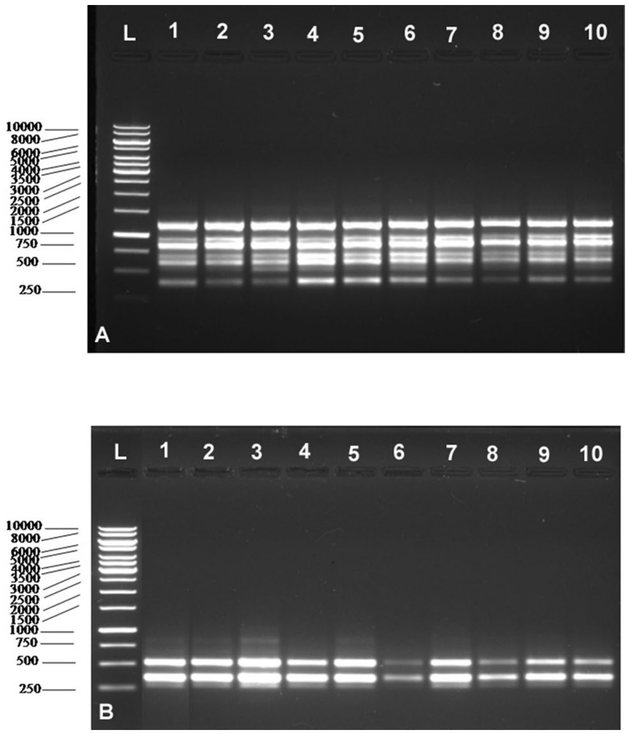

Fig. 3 RAPD banding profile among regenerants. **a** Primer OPA-10. **b** Primer OPC-13 where L = ladder; 1–10 = regenerants

or conjugation with alanine that results in biological inactivation by forming chemically stable derivatives (Webster and Jones 1991; Sakakibara 2006). Therefore, investigations on the use of alternatives for BA in micropropagation of woody tree species are imperative. *Meta*-topolin has been used as a potential substitute of BA in several plants, such as *Spathiphyllum floribundum* (Werbrouck et al. 1996); *Barleria greenii* (Amoo et al. 2011) and *Prunus* stock (Gentile et al. 2014). *Meta*-topolin has been found to be more effective than BA at similar equimolar dose in the present study. Establishment of *O*-glucoside metabolites is possible through the hydroxyl group in the side chain of *m*T (Werbrouck et al. 1996). The *O*-glucosides are considered to be a storage form of cytokinins that are stable under certain situations and rapidly convertible to potential cytokinin bases whenever needed (Werbrouck et al. 1996). Higher rates of shoot formation may be because of alterable sequestration of the *O*-glucosides, which permits continual availability of cytokinins at a physiologically vital level over a prolonged period (Strnad 1997).

Several researchers reported in many plant species that a low dose of auxin is required in addition to cytokinin for improving shoot rate multiplication and proliferation (Ferguson and Beveridge 2009; Muller and Leyser 2011). In our study, a medium enriched with *m*T (7.5 µM) and NAA (1.0 µM) showed a synergetic effect in the improvement of the multiplication rate of shoots (17.44) per explant, after 12 weeks. This is in contrast with the results reported by Chand and Singh (2004) in *P. marsupium* where only 9.5 shoots per explant were obtained on a medium augmented with 4.44 µM BA and 0.26 µM NAA after 15 weeks of culture. The results clearly showed the use of *m*T as a potential alternate for BA in micropropagation of this woody tree.

Rooting efficiency of *m*T-derived microshoots was better than BA-derived shoots with a good survival rate of regenerants (80%) in ex vitro conditions, compared to a 67% survival rate with BA-regenerated microshoots (Table 2). Several aspects such as its better uptake, transportation, and permanence over other auxins and successive gene activation are responsible for the stimulatory effects of IBA on root formation (Ludwing-Muller 2000). We have observed a better acclimatization success of *m*T-derived plantlets compared to the BA derived. There are reports that have documented that the type and dose of cytokinins have a profound effect on in vitro acclimatization competence (Bairu et al. 2008; Valero-Aracama et al. 2010). It has been reported that BA has a negative effect on root formation and results in deprived acclimatization rates in many plant species (Werbrouck et al. 1995; Bairu et al. 2008).

The *m*T treatment resulted in improvement of quality, number of shoots, photosynthetic pigments and biomass content when compared to BA-treated plantlets. The possible reason for the improved effect of *m*T over BA may be its role in delay of senescence of leaves (Wojtania 2010), increase in chlorophyll content (Aremu et al. 2012), improved rate of photosynthesis and altering the source-sink distribution (Čatský et al. 1996). These traits appeared to have resulted in an enhanced total biomass of the plant.

During in vitro regeneration practices, the existence of somaclonal variations between sub-clones of an elite parental line is a potential disadvantage (Martin et al. 2004; Ahmed et al. 2017). Rahman and Rojara (2001) suggested that genetic homogeneity of in vitro-raised plantlets is a prerequisite to maintain the advantages of elite genotypes. Use of plant growth regulators at higher concentration and repeated subculturing of sub-clones for long periods in tissue culture systems hampers maintenance of genetic fidelity (Sahijaram et al. 2003). We have chosen a CN explant because of its high-regeneration efficiency for shoot multiplication compared to a nodal segment explant (Husain et al. 2008) and hypocotyl explant (Husain et al. 2010). Similarly, true-to-type clonal fidelity analysis from single CN-derived plantlets using various molecular marker techniques has been well documented in many species such as *Withania somnifera* (Nayak et al. 2013) and *Cassia alata* (Ahmed et al. 2017). Among the regenerated plantlets, no visible variation in morphology was observed. Further, genetic fidelity of regenerated plantlets was confirmed using RAPD marker analysis. The banding profile was similar among the regenerants. The monomorphic analysis of ten randomly selected regenerants exhibited complete genetic uniformity.

Conclusions

The findings of the study showed the positive effect of *m*T over the commonly used cytokinin (BA) in attaining multiple shoot production from CN explants with no abnormalities in *Pterocarpus marsupium*. Inclusion of *m*T in media may provide a method to ensure efficient commercial in vitro propagation of a large number of diverse genotypes. However, further screening of the efficacy of *m*T with a wide range of *Pterocarpus* genotypes is required to confirm the broader application.

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Author Contributions Experiment designed and executed by AA. The whole experiment was conducted under the supervision of MA who also read and edited the manuscript.

Compliance with Ethical Standards

Conflict of interest There is no conflict of interest.

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