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Micropropagation of *Terminalia bellerica* Roxb. from juvenile explants

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An *in vitro* micropropagation system has been developed for *Terminalia bellerica* Roxb., an important Indian medicinal plant. Nodal segments obtained from 15-d-old aseptically grown seedlings were used as explants. MS medium containing 1.5 mg L^{-1} BAP was found most suitable for culture initiation. Although shoot multiplication was achieved on MS medium containing BAP and Kn, the maximum number of shoots was obtained with 1.5 mg L^{-1} BAP. Best rooting response (60%) was observed on medium containing quarter strength MS salts, 0.6% agar and 0.1 mg L^{-1} IBA. Plantlets were hardened initially in culture room conditions and then transferred to misthouse.

Keywords: Axillary bud proliferation, Bahera, *in vitro*, rooting, shoot multiplication

Introduction

Terminalia bellerica Roxb., commonly known as 'Bahera', is a large tree belonging to family Combretaceae (Fig. 1). The fruits possess antioxidant properties and form an important ingredient of many ayurvedic preprations, such as 'Triphla'[1]. Conventional methods of multiplication of *T. bellerica* have proved inadequate on account of hard seed-coat, heavy insect infestation of seeds and low survival rate of cuttings[2] and, therefore, require alternative methods of propagation.

In recent years, plant tissue culture techniques have been employed for multiplication of various tree species using seedling and mature explants[3-7]. Micropropagation of *T. bellerica* using mature node has been earlier reported by Roy *et al*[2], while Bilochi[8] was able to establish shoot cultures using seedling nodes. However, the efficiency of multiplication was reported significantly low. Present investigation was carried out to develop an efficient *in vitro* micropropagation protocol for *T. bellerica* from aseptically raised seedling explants.

Materials and Methods

The fruits of *T. bellerica* were collected from natural population growing around Udaipur, Rajasthan. They were dried and seeds were taken out after breaking the hard seed-coat. The seeds were surface sterilized with 0.15% $HgCl_2$ for 15 min. After rinsing for 5-6 times with autoclaved distilled water, they were inoculated aseptically on to water agar (0.8%) for germination. Cotyledonary and epicotyledonary nodes obtained from 15-d-old seedlings were implanted vertically on to different culture media [Murashige and Skoog[9] (MS), Schenk and Hildebrant[10] (SH), Woody Plant Medium[11] (WPM), Gamborg *et al*[12] (B_5), and White's[13]] containing 1.5 mg L^{-1} BAP. Different concentrations of BAP and Kn (0.5-5.0 mg L^{-1}) were used individually for proliferation of shoots from seedling nodes. Auxins (NAA & IAA) were combined with optimum BAP concentration for shoot proliferation. Same experiments were repeated for shoot multiplication.

The medium containing 3% sucrose was solidified with 0.8% agar (Hi-media). The *p*H of the media was adjusted to 5.8±0.02 with 1 N NaOH or 0.5 N HCl solution prior to autoclaving. Media poured in culture vessels were steam sterilized by autoclaving at 1.06 Kg cm^{-2} for 15-20 min. The cultures were incubated under controlled conditions of temperature (28±2°C), light (45 μmol m^{-2} s^{-1} for 16 h/d provided by fluorescent tubes) and 60-70% humidity.

For each experiment a minimum of 5 replicates were taken and experiments were repeated thrice. Observations were recorded after an interval of 3 wk. Once culture conditions for shoot induction from explants were established, the shoots produced *in vitro* were subcultured on fresh medium every 3 wk. Rooting of elongated shoots was attempted

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Abbreviations: BAP: 6-benzylaminopurine; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; Kn: kinetin; NAA: 1-naphthalene acetic acid.

under *in vitro* conditions. Auxins (IAA, IBA & NAA) alone or in combination in different concentrations (0.1-5.0 mg L^{-1}) were incorporated in the agar (0.6%) solidified medium containing 1/4 MS salts and 1.0% sucrose.

The *in vitro*-rooted plantlets were transferred to culture bottles 1/4th filled with autoclaved Soilrite^{TM} and irrigated with 1/4 MS salt solution. These bottles were kept in controlled environmental conditions of culture room. After 3 wk of growth, the plantlets were transferred to misthouse for further growth.

Results

The epicotyledonary and cotyledonary (after removal of cotyledons) nodes when inoculated on MS medium containing BAP and Kn in the range 0.5-5.0 mg L^{-1} showed enhanced shoot proliferation. BAP at its 1.5 mg L^{-1} concentration evoked best response. Incorporation of NAA or IAA improved bud proliferation but the shoots remained stunted. When explants were inoculated on various media containing 1.5 mg L^{-1} BAP, MS medium elicited best response, followed by SH, WPM, B_5 and White's medium.

Shoots after their initial proliferation on medium containing 1.5 mg L^{-1} BAP were subcultured on same fresh medium after every 21 d (Fig. 2). When shoot clusters were inoculated on various media for multiplication, the maximum number of shoots (10.6) was obtained on SH medium but stunting and yellowing of shoots were observed, which intensified on subsequent subcultures on the same fresh medium. Better growth of shoots was, however, obtained on MS medium and average shoot length remained highest on it (Table 1). All other media were found unsuitable for shoot multiplication. Consequently, in all subsequent experiments, MS medium was used.

Figs (1-4)—1. A mature tree of *T. bellerica* growing in natural habitat, 2. Multiplying shoots of *T. bellerica* grown on MS medium containing 1.5 mg L^{-1} BAP, 3. In vitro root induction in *T. bellerica* on ¼ MS medium containing 0.1 mg L^{-1} IBA, and 4. 42-d-old hardened plants of *T. bellerica* growing on Soilrite^{TM} moistened with ¼ MS salt solution.

Incorporation of BAP or Kn into MS medium supported multiplication of shoots in culture. BAP proved to be a better choice than Kn and the maximum number of shoots was obtained on its 1.5 mg L^{-1} concentration (Table 2). When NAA or IAA was used in combination with BAP (1.5 mg L^{-1}), a variety of responses were observed. At higher concentrations (0.5 mg L^{-1} and above) of both the auxins, callusing was observed. Shoot multiplication was improved in presence of 0.5 mg L^{-1} NAA, and 0.25 and 0.5 mg L^{-1} IAA (Table 2), combined with 1.5 mg L^{-1} BAP. However, in presence of auxins, shoots remained stunted and the length did not improve even after repeated subculture on the same fresh medium. Therefore, for shoot multiplication, MS medium containing 1.5 mg L^{-1} BAP was considered most appropriate.

Auxins (IAA, IBA & NAA) in different concentrations (0.1-1.0 mg L^{-1}) induced rooting when incorporated in the medium containing 1/4 MS salts (Table 3). Callusing was observed on all auxins used at their higher concentrations (0.5 mg L^{-1} and above). Best rooting response (60%), however, was observed on medium containing 0.1 mg L^{-1} IBA where 1.36

Table 1—Effect of various nutrient media on shoot multiplication in *T. bellerica*

Media	Av. no. of shoots	Av. length of shoots (cm)
MS	9.00 ± 0.71^{b}	1.70 ± 0.14^{a}
SH	10.60 ± 0.55^{a}	0.90 ± 0.14^{b}
WPM	8.40 ± 1.14^{b}	$0.80\pm0.27b^{c}$
B_5	4.00 ± 0.00^{c}	$0.76\pm0.13b^{c}$
BTM	4.60 ± 1.34^{c}	$0.64\pm0.13c^{d}$
WM	4.00 ± 0.00^{c}	0.50 ± 0.00^{d}
SE	0.361	0.070
CV	23.23	17.96
CD [5%]	0.35	1.052

Table 2—Effect of PGRs on shoot multiplication in *T. bellerica* grown on MS medium

PGRs \ Conc. (mg L^{-1})		No. of shoots*						Shoot length# (cm)					
		0.0	0.5	1.0	1.5	2.0	5.0	0.0	0.5	1.0	1.5	2.0	5.0
BAP		3.00±0.00^{f}	5.00±1.22^{ef}	5.60±0.55^{e}	9.00±0.71^{bc}	7.00±1.58^{d}	3.00±0.00^{f}	0.50±.00^{ef}	0.76±0.11^{d}	0.98±0.04^{c}	1.70±0.14^{a}	0.88±0.08^{cd}	0.50±0.07^{ef}
Kn		3.00±0.00^{f}	4.00±1.00^{f}	4.60±0.55^{ef}	5.20±0.84^{ef}	6.00±1.58^{de}	8.00±1.58^{cd}	0.50±.00^{ef}	0.66±0.15d^{e}	0.68±0.13^{d}	0.96±0.18^{c}	1.26±0.32^{b}	1.54±0.32^{a}
Cytokinin	Auxin	0.0	0.10	0.25	0.50	0.75	1.0	0.0	0.10	0.25	0.50	0.75	1.0
BAP	NAA	9.00±0.71^{bc}	8.60±0.89^{c}	9.40±0.55^{bc}	13.20±0.84^{a}	4.20±0.45^{f}	3.60±0.55^{f}	1.70±0.14^{a}	0.50±0.00^{ef}	0.48±0.04^{f}	0.48±0.04^{f}	0.44±0.99^{fg}	0.40±0.00^{fg}
(1.5 mg L^{-1})	IAA	9.00±0.71^{bc}	5.80±1.48^{d}	10.20±2.07^{b}	12.80±0.84^{a}	6.40±0.89^{de}	4.00±0.00^{f}	1.70±0.14^{a}	0.40±0.00^{fg}	0.50±0.00^{ef}	0.68±0.16^{d}	0.48±0.04^{f}	0.30±0.00^{g}

*SE=0.459, *CV=15.50, *CD [5%] =1.290 #SE=0.059, #CV=18.41, #CD [5%] =0.166

All data represent average of five replicates

Table 3—Effect of various auxins on rooting in *T. bellerica* on 1/4 strength MS medium

PGRs \ Conc. (mg L^{-1})		No. of roots*					Root length# (cm)				
		0.1	0.25	0.5	0.75	1.0	0.0	0.5	1.0	1.5	2.0
IBA		1.31±0.31^{abc}	1.38±0.37^{abc}	1.23±0.23^{bc}	1.35±0.48^{abc}	1.23±0.33^{abc}	1.54±0.49^{a}	1.20±0.20^{bcde}	1.09±0.12^{ef}	1.09±0.12^{ef}	1.09±0.12^{ef}
IAA		1.08±0.19^{c}	1.31±0.31^{bc}	1.31±0.31^{abc}	1.31±0.31^{abc}	1.31±0.31^{abc}	1.12±0.26^{ef}	1.47±0.44^{ab}	1.44±0.40^{abcd}	1.38±0.36^{abcde}	1.18±0.19^{bcdef}
NAA		1.08±0.19^{c}	1.23±0.33^{bc}	1.15±0.33^{c}	1.15±0.33^{c}	1.15±0.33^{c}	1.15±0.33^{cdef}	1.17±0.23^{bcdef}	1.04±0.10^{f}	1.04±0.10^{f}	1.03±0.06^{f}
	Auxin	0.1		0.5		1.0	0.1		0.5		1.0
IBA	NAA	1.69±0.41^{a}		1.59±0.57^{ab}		1.38±0.37^{abc}	1.33±0.19^{abcdef}		1.13±0.12^{def}		1.08±0.08^{ef}
(0.1 mg L^{-1})	IAA	1.38±0.37^{abc}		1.33±0.19^{abc}		1.33±0.19^{abc}	1.13±0.12^{def}		1.33±0.19^{abcdef}		1.46±0.30^{abc}
		*SE=0.152		*CV=26.10		*CD [5%] =0.166	#SE=0.111		#CV=20.42		#CD [5%] =0.312

All data represent average of five replicates

roots measuring 2.62 cm (average) were formed (Fig. 3). Combining IBA (0.1 mg L^{-1}) with NAA or IAA increased the percentage of rooting but the roots formed were short, thick and without laterals. Elongation of shoots was not accompanied with rooting in any of the experiments.

In vitro rooted plantlets were initially hardened in culture room conditions where leaves expanded. After 3 wk, the plantlets were shifted to misthouse. There was an increase in length of shoots and new leaves emerged which expanded quickly (Fig. 4).

Discussion

Seedling derived explants, being juvenile, are frequently used for micropropagation as they are easy to establish in culture[14]. The most widely used method of *in vitro* plant propagation is the stimulation of axillary bud development. In presence of cytokinin/s, bud dormancy is broken and axillary branches proliferate[15]. In *T. bellerica*, MS medium containing 1.5 mg L^{-1} BAP was the best for culture initiation. MS medium has been frequently used for micropropagation of large number of plants[7]. We have found that *T. bellerica* cultures grew better on MS medium in comparison to other media. Cytokinins are believed to induce bud break and shoot proliferation[16]. In *T. bellerica*, 1.5 mg L^{-1} BAP was most suitable for shoot multiplication. Enhanced shoot multiplication by addition of auxin along with cytokinin has been reported in some plants[17-19]. We also observed improvement in shoot multiplication by incorporation of NAA (0.5 mg L^{-1}) and IAA (0.25 and 0 .5 mg L^{-1}) in the medium along with BAP (1.5 mg L^{-1}) but the shoots remained stunted and the length did not improve even on repeated subculture of shoots on the same fresh medium.

IBA has been widely used as root induction hormone under *in vitro* and *in vivo* conditions[20]. We also found positive role of IBA during *in vitro* rooting. In *T. bellerica*, 0.1 mg L^{-1} IBA proved to be the best for *in vitro* rooting. The *in vitro* rooted plants were hardened first under controlled conditions of culture room and then shifted to misthouse where they exhibited enhanced growth and 100% survival.

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