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Rapid micropropagation and callus induction of *Terminalia bellerica* Roxb. - An endangered plant

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ABSTRACT

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 2.0 mg/l 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 3.5 mg/l BAP+ 0.5 mg/l Kn. Best rooting response was observed on medium containing quarter strength MS salts, 0.8% agar and 1.0 mg/l IBA. Plantlets were hardened initially in culture room conditions and then transferred to misthouse. Maximum callus induction response was observed on MS medium supplemented with 0.25 mg/l 2,4-D+ 0.3 mg/l Kn within 4 weeks from leaf petiole.

Keywords: Medicinal plant, callus, Bahera, micropropagation, shoot multiplication.

INTRODUCTION

Plants containing beneficial and medicinal properties have been known and used as sources of food, fodder, oils, medicines, fuel, wood, fibers, and timber by increasing population growth. Due to increased demand for pulp, paper, construction materials, farmlands and fuel, status of woody trees especially forest trees are greatly affected (Giri et al. 2004). Plant is our natural wealth and its conservation is important for economic, ecological, scientific, medicinal and ethical reasons. Therefore, there is a great need to conserve forest ecosystems by agro-technology. In recent years, *in vitro* approaches have been used as an efficient tool for micropropagation of trees and it proved that tissue culture technology is suitable for large-scale propagation of trees in short time (Pena and Seguin 2001). Propagation of woody trees through tissue culture has many advantages over conventional propagation method like fast multiplication of the important genotypes, quick release of improved cultivars, production of disease-free plants, season-independent production of plants, germplasm conservation and facilitating their easy exchange (Asthana et al. 2011).

Terminalia bellerica Roxb. (Family Combretaceae) commonly known as 'Bahera', is a perennial deciduous tree of the tropics and found in south Asia, including Aravalis in Rajasthan, India. The fruits possess antioxidant, antistressor and immunostimulant properties and form an important ingredient of many ayurvedic preprations, such as **'Triphla'**. 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 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. Micropropagation of *T. bellerica* using mature node has

been earlier reported by Roy *et al*, (1987) while Bilochi 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 protocol for micropropagation of *T. bellerica* from nodal explants of seedling.

MATERIALS AND METHODS

The fruits of *T. bellerica* Roxb. were collected from garden of Ummed Bhawan Place Kota, Rajasthan. They were dried and seeds were taken out after breaking the hard seed-coat. The seeds were surface sterilized subsequently with running tap water, labolin soap solution and finally with 0.1% $HgCl_2$ for 15 min under laminar bench. 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 (MS)] containing 2.0 mg/l BAP. Different concentrations of BAP and Kn (1.0-5.0 mg/l) were used individually for proliferation of shoots from seedling nodes. Combination of BAP and Kn were used for shoot proliferation. Same experiments were repeated for shoot multiplication.

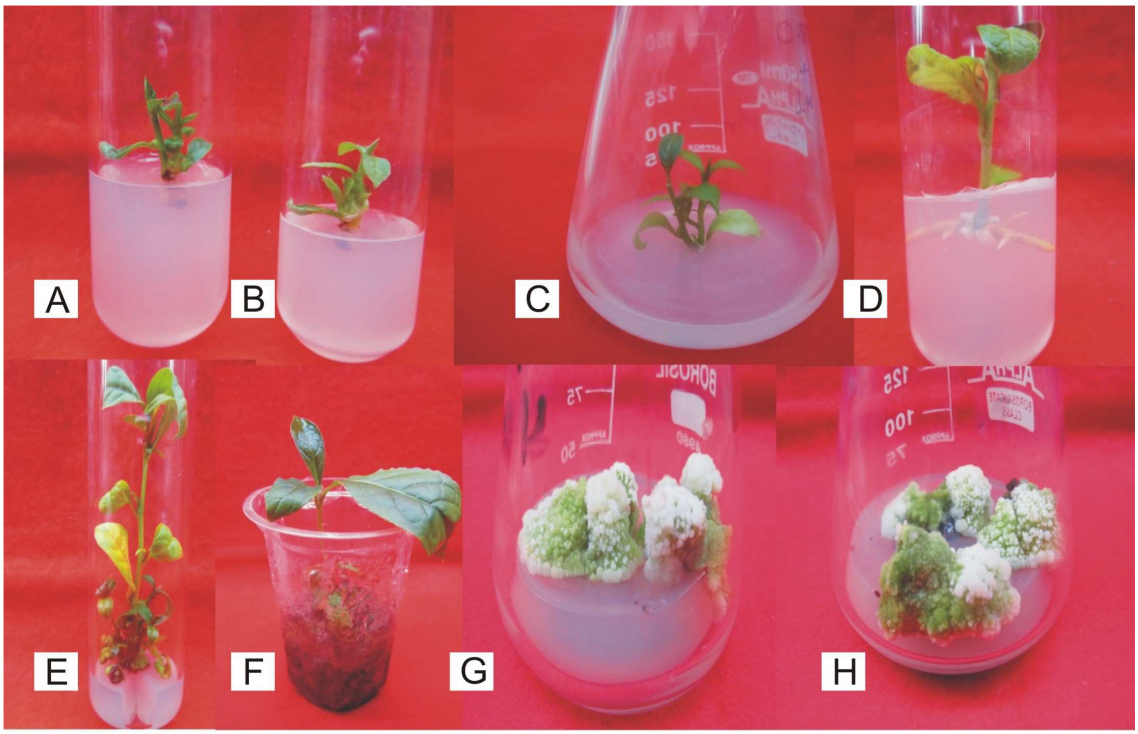
The medium containing 3% sucrose was solidified with 0.8% agar (Qualigens). The *p*H of the media was adjusted to 5.9+0.02 with 1 N NaOH or 1 N HCl solutions prior to autoclaving. Media poured in culture vessels were steam sterilized by autoclaving at 15 lbs for 15-20 min. The cultures were incubated under controlled conditions of temperature (25±2°C), light (2000- 2500 lux for 16 h/d provided by fluorescent tubes) and 60-70% humidity. For each experiment a minimum of 7 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 sub cultured on fresh medium every 3 wk. Different combination of Auxins (0.25 mg/l) (2, 4-D) and Cytokinine (BAP and Kn) (0.1-0.3 mg/l) were used for callus induction from leaf petiole. Rooting of elongated shoots was attempted under *in vitro* conditions. Auxins (IBA) alone in different concentrations (0.5-2.5mg/l) were incorporated in the agar (0.8%) 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 Soilrite composition (soil: sand: peat moss) 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 AND DISCUSSION

The epicotyledonary and cotyledonary (after removal of cotyledons) nodes when inoculated on MS medium containing BAP and Kn in the range 1.0-5.0 mg/l showed enhanced shoot proliferation. BAP at its 2.0mg/l concentration evoked best response. Incorporation of NAA or IAA improved bud proliferation but the shoots remained stunted. Shoots after their initial proliferation on medium containing 20mg/l BAP were sub-cultured on same fresh medium after every 21 days. 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 shoot was obtained on its 2.0 mg/l concentration (Table 1, Fig. A-B). When BAP was used in combination with Kn (0.5mg/l), a variety of responses were observed (Table 2, Fig. C). But best response was observed on medium containing 3.5 mg/l BAP + 0.5 mg/l Kn (Average number of shoots 5.02±0.76) and best shoot length was observed on medium containing 2.0 mg/l BAP+ 0.5 mg/l Kn (Average shoot length 3.87±0.39 cm). The full or half strength of MS medium without any PGR was failed to induce rooting of regenerated shoots. However, shoots were capable to induce root when cultured on medium containing auxins.

Auxins in different concentration induced rooting when incorporated in the medium containing ¼ of MS salts. The best rooting response, however, was observed on medium containing 1.0mg/l IBA, where roots measuring 1.68±0.32 cm (average) were formed (Table 3, Fig. D). *In vitro* rooted plantlets were initially hardened in culture room conditions where leaves expanded. After 3 week, the plantlets were shifted to mist house. There was an increase in length of shoots and new leaves emerged which expanded quickly (Fig. E-F). Leaf petiole explants were used for the purpose of callus induction. Highest diameter of callus was observed on MS Medium fortified with 0.25 mg/l 2,4-D+ 0.1 mg/l BAP (callus diameter 3.5 cm) and 0.25 mg/l 2,4-D+ 0.3mg/l Kn (callus diameter 3.8 cm), (Table 4, Fig. G-H).

Fig. 1 (A-H) Clonal micropropagation of *T.bellrica* from nodal shoot explant



A. Shoot multiplication on MS medium Supplemented with 2.0 mg/l BAP B. Shoot multiplication on MS medium Supplemented with 5.0 mg/l Kn C. Shoot multiplication on MS medium Supplemented with 3.5 mg/l BAP+0.5mg/l Kn D. In vitro root induction on ¼ MS medium+ 1.0 mg/l IBA E. Plant developed from In vitro rooting F. 7 week old hardened plant growing on soilrite moistened with basal MS medium. G. Callus induction from leaf petiole on MS medium Supplemented with 0.1 mg/l BAP+0.25 mg/l 2,4-D H. Callus induction from leaf petiole on MS medium Supplemented with 0.3 mg/l Kn+0.25 mg/l 2,4-D.

Table 1. Effect of Cytokinin (BAP and Kn) on shoot proliferation from nodal shoot explant of *T.bellrica*

Hormone Con. (mg/ l) BAP	Hormone Con. (mg/ l) Kn	Response (%)	No. of Shoot/explant (mean±SD)	Shoot length (in cm) (mean±SD)
1.0	-	30	0.80±0.41	0.52±0.04
2.0	-	80	3.80±0.77	2.04±0.59
3.0	-	60	2.40±0.51	1.38±0.12
4.0	-	55	1.00±0.51	0.88±0.10
5.0	-	40	0.50±0.07	0.82±0.70
-	1.0	0	-	-
-	2.0	20	0.60±0.50	0.70±0.25
-	3.0	35	0.96±0.18	0.80±0.28
-	4.0	55	1.26±0.32	1.38±0.98
-	5.0	60	1.54±0.31	1.82±0.27

Medium: MS+ additives; mean± SD, n= 7 replicates
Means having the same letter in each Colum, do not different significantly at P< 0.05 (Tukey's test)

Table 2. Interactive effect of Cytokinin (BAP+ Kn) on shoot multiplication by sub culture of shoot clumps of *T.bellrica*

Hormone Con. (mg/ l)	No. of Shoot/explant	Shoot length (in cm)	Shooting Response (%)
0.5 BAP + 0.5 Kn	1.19±0.21	1.44±0.80	64
1.0 BAP + 0.5 Kn	1.52±0.15	1.70±0.14	67
1.5 BAP + 0.5 Kn	2.26±0.24	2.60±0.51	74
2.0 BAP + 0.5 Kn	2.31±0.48	3.87±0.39	76
2.5 BAP + 0.5 Kn	3.78±0.57	3.06±0.22	80
3.0 BAP + 0.5 Kn	4..98±0.74	2.84±0.98	87
3.5 BAP + 0.5 Kn	5.02±0.76	2.17±0.47	90

Medium: MS+ additives; mean±SD, n= 7 replicates
Means having the same letter in each Colum, do not different significantly at P< 0.05 (Tukey's test)

Table 3. Effect of Auxin (IBA) on root induction from isolated shoot of *T.bellrica*

Hormone Con. (mg/ l)	No. of roots/explants	Root length (in cm)	Rooting Response (%)
0.5 IBA	2.80±0.73	0.43±0.33	68
1.0 IBA	3.60±0.51	1.68±0.32	90
1.5 IBA	1.40±0.52	1.06±0.08	82
2.0 IBA	1.38±0.37	0.92±0.10	78
2.5 IBA	1.08±0.19	0.51±0.05	73

Medium: MS+ additives; mean±SD, n= 7 replicates
Means having the same letter in each Colum do not different significantly at P< 0.05 (Tukey's test)

Table 4. Effect of different Hormones on Callus proliferation and Morphology

Hormone Conc. (mg/l)	Callus diameter after 7 weeks subculture (cm)	Callus proliferation Scoring	Color of callus	Morphology of callus
2,4-D + Kn	3.2	+++	Green	Compact
0.25 + 0.1	2.6	++	Whitish green	Friable
0.25 + 0.2	3.8	++++	Dark green	Nodular
0.25 + 0.3				
2,4-D + BAP				
0.25 + 0.1	3.5	++++	Dark green	Nodular
0.25 + 0.2	2.8	++	Whitish green	Friable
0.25 + 0.3	2.4	+++	Green	Compact

'++++' *Intense*
'+++' *Moderate*
'++' *Meager*

CONCLUSION

The seedling derived explants, being juvenile, are frequently used for micropropagation, as they are easy to establish in culture. In *T. bellerica*, MS medium containing 2.0mg/l BAP was the best for culture initiation. We have found that *T. bellerica* culture grew better on MS medium in comparison to other media. In *T. bellerica*, 2.0 mg/l BAP was most suitable for shoot multiplication. We also observed improvement in shoot multiplication by different concentrations of BAP (0.5-3.5 mg/l) in medium along with Kn (0.5 mg/l). Best shooting response was observed on media containing 3.5 mg/l BAP+ 0.5 mg/l Kn (Average number of shoots 5.02) and 2 mg/l BAP+ 0.5 mg/l Kn (Average shoot length 3.87 cm).

IBA (Auxin) has been widely used as root induction hormone under *in vitro* and *in vivo* condition. We also found positive role of IBA during in vitro rooting. In *T. bellerica*, 1.0mg/l 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 mist house where they exhibited and hence, growth and 90% survival. Most responsive callus induction was observed on MS Medium supplemented with 0.25 mg/l 2,4-D+ 0.1 mg/l BAP.

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