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In Vitro Plantlets Regeneration of *Terminalia bellirica Roxb.* An important Medicinal Tree

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Abstract

Rapid in vitro micropropagation protocol of T. bellirica was achieved using nodal explant from mature tree. Nodal explants when placed on Murashige and Skoog medium supplemented with 0.5 mgl^{-1} 6-benzylaminopurine showed 100% shoot-bud with 4.5±0.56 shoot length per explant. The nodal segments from micro shoot obtain from induction medium were cultured on MS basal medium supplemented with different concentration of BAP and NAA, best shoot multiplication occurred with 0.25 mgl^{-1} BAP + 0.25 mgl^{-1} NAA. The shoot and node number increased significantly at third and fourth subculture of nodal segments. Excised shoots (2 cm - 3 cm long with 2 to 3 nodes) when grown on half MS basal medium with 0.25 mgl^{-1} indole-3-butyric acid (IBA) and pulse has shown rhizogenesis. The plantlets were washed to remove agar and then planted root trainers containing soil rite or sand soil mixture as substrate. These cultures were placed in green house for primary hardening. After four-weeks, plants were transferred from green house to net house where the plants exhibited gradual acclimatization to outdoor conditions.

Keywords: *Terminalia bellirica*; Murashige and Skoog medium; Rhizogenesis; micropropagation; micro node, BAP, NAA.

Introduction

The impact of population explosion on the forest wealth of India has been disastrous. Consequently, the number of species facing extinction has increased due to disturbance in their natural habitat, the genetic diversity of man species, in their natural population has shrunk, the total area under tree cover has reduced significantly and a large area of existing forest has degraded. Now efforts are afoot to preserve endangered species, conserve genetic diversity and arrest forest degradation. The yield of plantation can be enhanced by using genetically superior cultivars. In India, tree improvement programmed by selection and breeding started a few decades ago. Due to long life cycle of trees, breedings methods have only a limited scope. Therefore, in vitro micropropagation method are viewed as potent supplement to the traditional methods of plant improvement.

T. bellirica also known as, Beleric Myrobalan in English, Bibhitaki in Sanskrit, Locally known as Bahera in India, It is found in abundance in Madhya Pradesh, Uttar Pradesh, Punjab, Maharashtra states of India and also in Sri Lanka and Malaya[1]. Bahera is a large deciduous tree with bluish or ashy - grey bark and about 20- 25 m hight[2]. Bahara is an immensely useful medicinal tree. Its several parts are used to cure variety of diseases. The fruit rind (pericarp) is astringent, laxative, anthelmintic, pungent, antipyretic and Rejuvenating[3]. It is applied in a diverse range of conditions including cough, tuberculosis, eye diseases, anti-HIV-1, dyspepsia, diarrhoea, dysentery, biliousness, flatulence, liver disease, leprosy, cleanse the blood and promote hair growth.

Material and Methods

Explants sterilization and establishment: Explants were taken from mature tree of *T.bellirica* grown at the botanical garden of Pt. Ravishankar University, Raipur. Nodal segment were cut into 1 to 2 cm length along with node. All the explants were washed in tap water and then leboline (0.2%). Following a 5 min. Sterilized treatment in 0.2% (w/v) aqueous solution $HgCl_2$ and then washed 4-5 times in sterilized distilled water. The sterilized nodes were inoculated on MS[4] fortified with multiple concentration of 6-benzylaminopurine (0.25, 0.5, 1.0, 2.0, 4.0 mgl^{-1}) and Kinetin (0.25, 0.5, 1.0, 2.0, 4.0 mgl^{-1}). Similarly sterilized nodes were inoculated on WPM[5] and SH[6] and B_5[7] medium each fortified with 0.5 mgl^{-1} 6-benzylaminopurine, for explants establishment. The pH of all the media was adjusted to 5.6-5.8 using 1N NaOH or 1N HCl after mixing all the constituents except the gelling agent.

Shoot proliferation and subculture: After 4 weeks, the micro-nodes of micro shoots developed from explants on establishment medium were placed on shoot multiplication medium (MS medium) contained with different concentrations of BAP (0.25, 0.5, 1.0 and 2.0 mgl^{-1}) and BAP + NAA (0.25+0.25, 0.25+0.5, 0.25+1.0 and 0.25+2.0 mgl^{-1}) for shoot proliferation. Four-weeks-old cultures on shoot multiplication medium MS with 0.25 mgl^{-1} BAP + 0.25 mgl^{-1} NAA served as source of nodes for subculture. Every 4th week, the nodal segments were harvested and serially sub-cultured upto five cycles in same medium to find out achieve of subculture on shoot multiplication.

Rooting: The micro-shoots developed after fifth subculture were inoculated on half strength MS medium containing multiple concentrations of IBA (0.0, 0.06, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mgl^{-1}) to study the effect of IBA on in vitro rooting.

Acclimatization: In vitro rooted plantlets of *T.bellirica* were removed carefully from culture medium and washed under running tap water to remove agar. Plantlets were dipped in fungicide (0.1 % Bavistin) for 10 minutes and transplanted into root trainers containing soil-rite for primary hardening. After 4 weeks plants were shifted into nursery begs containing mixture of sand, soil (1:1) for secondary hardening.

Each experiment consisted of 10 replicates and all experiments were repeated 3 times. Data for explants establishment, shoot multiplication and rooting were collected after 4 weeks. The data was analysed by using one or two-way analysis of variance (ANOVA), standard error (SE) was plotted after means. Mean comparisons were made by least significant difference at the 5% probability level.

Results and Discussion

Effect of BAP and Kinetin: Nodal segments of mature trees were inoculated on MS medium containing multiple concentrations (0.0, 0.25, 0.5, 1.0, 2.0 and 4.0 mgl^{-1}) of BAP (table 1) and (0.0, 0.25, 0.5, 1.0, 2.0 and 4.0 mgl^{-1}) Kinetin (table 2). The nodal pieces placed on medium with BAP (0.5 mgl^{-1}) showed 100 % shoot bud initiation, 3.0 ± 0.42 shoot per explants, 4.0 ± 0.56 cm length per shoot and 3.0 ± 0.44 nodes per shoot (table 1, Figure A). These results suggest that 0.5 mgl^{-1} BAP in MS medium was suitable medium for explants establishment of mature trees of *T. bellirica*. The endogenous level of cytokinin or their precursor in the mature tree explants was high because of this required low amount of exogenous cytokinin for explants establishment. The effect of BAP for in vitro shoot formation in tree was earlier reported[8]. Mehta et al.[9] reported that MS with 3.0 mg/l is best medium for culture initiation in *Stevia rebaudiana* compara to other media. Ndoya et al.[10] found 2.5mgl^{-1} BAP was optimal for establishment of axillary bud explants from *Balanites aegyptiaca.* Pandey et al.[11] reported cytokinins were best medium for shoot multiplication from nodal explants of *T. arjuna* tree. Das and Pal[12] used MS medium and BAP for axillary bud induction.

Effect of Basal medium: Nodal segment were inoculated on MS medium, SH medium, WPM medium and B_5 medium contained with 0.5 mgl^{-1} BAP. The maximum percentage of shoot bud initiation (100%) occurred in MS medium (table 3). Thus, MS medium contained with BAP (0.5 mgl^{-1}) was found to be most suitable medium for establishment of *T.bellirica*. Same as Rajeswari and Paliwal[13] reported in *Pterocarpus santalinus*, the cotyledonary nodes showed best response in full strength MS medium when compared to WPM and B_5 medium. In earlier studied also MS medium was found best medium for in vitro establishment of explants from hardwood trees such as *Terminalia arjuna*[14], *Tectona grandis*[15], *Gmelina arborea*[16] and woody climber *Bauhinia vahlii*[17].

Shoot proliferation and Sub-culture: The micro nodes of shoots elongated from explants of mature tree on explants establishment medium MS with 0.5 mgl^{-1} were inoculated on shoot proliferation medium MS with multiple concentrations of BAP or combination of BAP and NAA. The micro nodes placed with 0.25 mgl^{-1} BAP + 0.25 mgl^{-1} NAA showed better response than other concentrations of BAP and combinations of BAP and NAA. The micro node placed on MS medium contained with 0.25 mgl^{-1} BAP + 0.25 mgl^{-1} NAA showed maximum shoots number per micro node 6.5 ± 0.62, length per shoot 5.5±0.45, nodes per shoot 4.5±0.82 (table 4, figure B). The micro node showed more number of shoots on proliferation medium have in lower concentration of BAP than the concentration of BAP in establishment medium. The low concentration of BAP required NAA for shoot multiplication. Usually, the shoots grown on medium with high concentration of cytokinin possess residual BAP within them that has inhibitory effect at shoot proliferation stage. In such cases lowering the concentration of BAP at proliferation stage has beneficial effect. Addition of auxin usually supports cultures at multiplication stages. Same as addition of auxin at low levels with cytokinin has to increase shoot number and also shoot elongation in other plant like *Sapium sebiferum*[18], *Nycanthes arbor*[19], *Mucuna puriens*[20]. Eman[21] also reported that shoot proliferation rate is high when cyotkinin along with low concentration of auxin.

The micro nodes were sub-cultured for five cycles. In first subculture, the micro nodes were placed on MS medium containing 0.25 mgl^{-1} BAP + 0.25 mgl^{-1} NAA and in subsequent second to fifth sub cultures, the micro nodes were grown on MS medium with same concentration of BAP and NAA as first cycle. Gradual increase in shoot length and number occurred up to 5th cycle (table 5, figure C).

Rooting of micro shoot: For rooting, 2-3 cm long micro shoots produced in vitro from explants of *T.bellirica* were placed on the rooting medium and given 24 hours, 48 hours and 72 hours dark pulse treatment. After the dark pulse treatment micro shoots were placed on MS medium without hormone. The 72 hours dark pulse treatment showed maximum root initiation 60%, maximum root number 2.0 ± 0.73 and maximum root length (cm) per micro shoot 3.6 ± 0.63 in micro shoot (table 6, figure D). Ishii[22] obtain rooting on regenerated when cultured on ½ MS medium with 1 uM IBA. Talukdar[23]also used half MS medium for in vitro rooting of *Adenium multiflorum* Ahirwar[24] reported that high concentration of IBA was better than low concentration of IBA for growth performance of seedling of *Alangium lamarckii*. Shadparvar[25] also showed that the proper concentration of IBA was useful for micropropagation of Cymbidium orchid.

Acclimatization of regenerated plants: The tissue culture raised plants were shift to root trainer and placed in green house at 70% humidity. In vitro regenerated plantlets *T. bellirica* were washed thoroughly to remove agar and transferred to root trainer containing different substratum. The root trainers were filled with soil rite or mixture of sand and soil (ratio 1:1) (figure

E). The plantlets transferred to root trainer filled with soil rite showed 80% survival and root trainer containing mixed soil and sand showed 60% survival. Secondary hardening was done in the nursery bage contaning soil mixture (Sand and soil in 1:1 ratio) and placed in a net house (figure F), where the plants grow on soil sand mixture showed 60 percent survival. Banerjee[26] reported that in vitro regenerated plantlets of *Bauhinia variegata* were transferred to plastic pots containing autoclaved soil showed 100% survival.

Table-1
The effects of different concentrations of BAP on shoot establishment from nodal segments

S.N.	Medium MS+ BAP (mgl^{-1})	Bud-break response (%)	Shoots/ explants Mean ± SE	Length (cm)/shoot Mean ± SE	Nodes/ Shoot Mean ± SE
1.	0.0	10	1.0 ± 0.24^{c}	1.5 ± 0.31^{d}	1.0 ± 0.46^{d}
2.	0.25	80	2.0 ± 0.33^{b}	3.5 ± 0.37^{b}	2.0 ± 0.45^{c}
3.	0.5	100	3.0 ± 0.42^{a}	4.0 ± 0.56^{a}	3.0 ± 0.44^{a}
4.	1.0	70	2.0 ± 0.51^{b}	2.8 ± 0.64^{bc}	2.5 ± 0.51^{b}
5.	2.0	50	1.5 ± 0.56^{bc}	2.5 ± 1.00^{bc}	2.0 ± 0.62^{c}
6.	4.0	50	1.0 ± 0.39^{c}	2.0 ± 0.56^{c}	1.5 ± 0.62^{d}

Mean ± 1 SEM; The means separated using DMR (Costat; Version 4.02), Similar alphabets within a column do not differ significantly at $P \leq 0.05$.

Table-2
The effects of different concentrations of kinetin on shoot establishment from nodal segments

S.N.	Medium MS+ Kin (mgl^{-1})	Bud-break response (%)	Shoots/ explants Mean ± SE	Length (cm)/shoot Mean ± SE	Nodes/ Shoot Mean ± SE
1.	0.0	10	1.0 ± 0.34^{c}	1.0 ± 0.35^{d}	1.0 ± 0.66^{c}
2.	0.25	70	2.0 ± 0.29^{b}	2.5 ± 0.29^{b}	2.0 ± 0.25^{b}
3.	0.5	80	2.5 ± 0.33^{a}	3.5 ± 0.51^{a}	3.0 ± 0.57^{a}
4.	1.0	60	2.0 ± 0.52^{b}	2.0 ± 0.54^{bc}	2.0 ± 0.76^{b}
5.	2.0	40	1.0 ± 0.36^{c}	2.0 ± 0.24^{bc}	1.5 ± 0.65^{bc}
6.	4.0	40	1.0 ± 0.47^{c}	1.5 ± 0.46^{c}	1.2 ± 0.56^{c}

Mean ± 1 SEM; The means separated using DMR (Costat; Version 4.02), Similar alphabets within a column do not differ significantly at $P \leq 0.05$.

Table-3
The effects of different media with BAP (0.5 mgl^{-1}) on shoot establishment from nodal segments

S.N.	Medium + 0.5 mgl^{-1}	Bud-break response (%)	Shoots/ explants Mean ± SE	Length (cm)/shoot Mean ± SE	Nodes/ Shoot Mean ± SE
1.	MS	100	3.0 ± 0.48^{a}	4.7 ± 0.76^{a}	4.0 ± 0.84^{a}
2.	SH	60	2.0 ± 0.47^{b}	3.0 ± 0.63^{b}	3.0 ± 0.58^{b}
3.	WPM	40	1.5 ± 0.42^{bc}	2.5 ± 0.55^{bc}	2.0 ± 0.39^{bc}
4.	B_5	20	1.0 ± 0.47^{c}	1.5 ± 0.58^{c}	1.0 ± 0.33^{c}

Mean ± 1 SEM. The means separated using DMR (Costat; Version 4.02), Similar alphabets within a column do not differ significantly at $P \leq 0.05$

Table-4
The shoot proliferation response of nodal segmets of microshoots derived from on establishments medium (MS with 0.5 mgl^{-1} BAP) and transferred to shoot proliferation medium

S.N.	MS +BAP (mgl^{-1})	MS+ BAP+NAA (mgl^{-1})	Shoots/ node Mean ± SE	Length/ Shoot Mean ± SE	Nodes/ shoot Mean ± SE
1.	0.5	0.0	2.0 ± 0.25^{d}	2.0 ± 0.38^{d}	2.0 ± 0.25^{d}
2.	0.5	0.25+0	6.0 ± 0.39^{ab}	5.0 ± 0.41^{ab}	4.0 ± 0.39^{ab}
3.	0.5	0.5+0	5.2 ± 0.47^{bc}	5.0 ± 0.67^{b}	4.0 ± 0.45^{ab}
4.	0.5	1.0+0	5.0 ± 0.39^{bc}	4.8 ± 0.65^{b}	3.5 ± 0.67^{abc}
5.	0.5	2.0+0	4.5 ± 0.33^{c}	3.3 ± 0.37^{c}	2.5 ± 0.63^{c}
6.	0.5	0.25 + 0.25	6.5 ± 0.62^{a}	5.5 ± 0.45^{a}	4.5 ± 0.82^{a}
7.	0.5	0.25+0.5	6.0 ± 0.39^{ab}	5.0 ± 0.32^{ab}	4.0 ± 0.38^{ab}
8.	0.5	0.25+1.0	5.5 ± 0.42^{b}	4.1 ± 0.54^{b}	3.0 ± 0.52^{b}
9.	0.5	0.25+2.0	4.5 ± 0.32^{c}	3.4 ± 0.67^{c}	2.8 ± 0.72^{b}
10.	0.5	0.5+0.25	5.5 ± 0.57^{b}	5.0 ± 0.45^{ab}	4.0 ± 0.57^{ab}
11.	0.5	0.5+0.5	5.0 ± 0.59^{bc}	4.0 ± 0.42^{b}	3.0 ± 0.53^{b}
12.	0.5	0.5+1.0	5.0 ± 0.50^{bc}	3.5 ± 0.45^{c}	3.0 ± 0.68^{b}
13.	0.5	0.5+2.0	4.8 ± 0.36^{c}	3.2 ± 0.43^{c}	2.9 ± 0.45^{b}

Table-5
Shoot proliferation during subcultures of nodes on MS with 0.25 mgl^{-1} BAP+0.25 mgl^{-1} NAA

Number of sub-culture	Shoots/node Mean ± SE	Length (cm) / shoot Mean ± SE	Nodes/shoot Mean ± SE
1.	4.0±0.54^{c}	5.0±0.73^{bc}	4.0±0.66^{c}
2.	4.5±0.67^{bc}	5.3±0.73^{b}	4.5±0.79^{b}
3.	5.3±0.76^{b}	5.7±0.70^{ab}	4.7±0.75^{ab}
4.	7.5 ±0.72^{a}	6.0±0.57^{a}	5.0±0.53^{a}
5.	4.0±0.51^{c}	4.0±0.82^{c}	3.0±0.51^{d}

Mean ± 1 SEM; The means separated using DMR (Costat; Version 4.02), Similar alphabets within a column do not differ significantly at $P \leq 0.05$

Table-6
Effects of IBA and dark pulse treatments on rooting of micro shoots derived from seedling explants

S.N.	IBA + dark pulse treatment (hr)	Root initiation response (%)	Roots / Shoot Mean ±SE	Root length (cm)/shoot Mean ± SE
1.	00	50^{c}	1.0±0.34^{b}	4.2± 0.50^{b}
2.	24	60^{b}	1.0±0.43^{b}	4.0± 0.57^{b}
3.	48	70^{ab}	1.0±0.33^{b}	4.6±0.65^{ab}
4.	72	80^{a}	2.2±0.54^{a}	5.0±0.64^{a}

Mean ± 1 SEM; The means separated using DMR (Costat; Version 4.02), Similar alphabets within a column do not differ significantly at $P \leq 0.05$


A


B


C

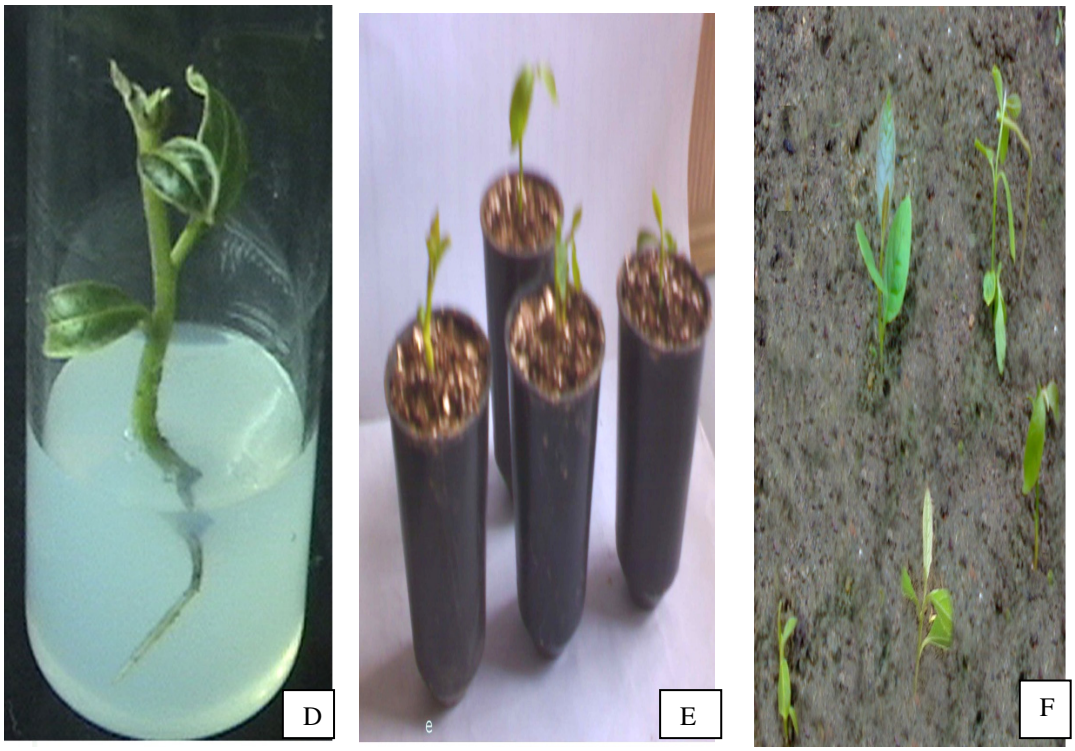

Figure-1
Shoot elongation from nodal segment placed on MS medium + 0.5 mgl^{-1} BAP (A); Shoot multiplication from micro nodes placed on MS medium + 0.25 mgl^{-1} BAP + 0.25 mgl^{-1} NAA (B); Sub-culturing of micro shoots (C), root on micro shoot placed on MS medium + 0.25 mgl^{-1} IBA and 72 pulse (D); hardening of plantlets in a net pot containing soil rite (E); transfer the plantlets into field (F)

Conclusion

It may be concluded that nodal explant of *Terminalia bellirica* required different level of hormone for each step of micropropagation. For induction they requires MS medium with 0.5 mgl^{-1} BAP, for multiplication they require cyokinine with low concentration of auxin (0.25 mgl^{-1} BAP + 0.25 mgl^{-1} NAA) and for rooting *T. bellirica* requires pulse treatment with IBA. By the end of the three phases it is possible to produce thousands of plantlets from single nodal explants. Therefore we developed a reliable protocol for in vitro propagation of *Terminalia bellirica*.

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