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In Vitro Conservation of *Tinospora formanii* Udayan and Pradeep-A Rare Endemic Plant from Western Ghats

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Abstract: *A successful protocol was developed for rapid clonal propagation of Tinospora formanii, a rare endemic medicinal plant of the family menispermaceae through the culture of mature nodal explants. Explants were disinfested with the use of 0.1% mercuric chloride and washed thoroughly with autoclaved distilled water. Solid MS medium was used with addition of different concentrations of 6-benzyl aminopurine (BAP) and indole butyric acid (IBA). The nodal segments inoculated in MS basal medium supplemented with BAP 2.0 mg L^{-1} and IBA0.5 mg L^{-1} initiated 2-3 buds in 6-7 days of culture. 95% of explants initiated shoots in this culture. Newly formed shoot clumps cultured on fresh medium with the same concentration of growth regulators as that of the induction medium resulted in shoot proliferation. An average of 12.75 shoots per explant was obtained after 30 days of culture. Individual shoots were elongated in MS medium supplemented with BAP 1.5 mg L^{-1} and IBA 0.5 mg L^{-1}. Half MS basal medium was found to be the best medium for rooting. Four to five strong healthy roots initiated in 7 days. Rooted micro shoots were successfully established in Soilrite-mix ^{TC} : garden soil (1:1) which was used as the hardening medium. The rooted explants were then gradually acclimatized and shifted to green house. Regenerated plantlets were successfully acclimatized and hardened off inside the culture room and then transferred to green house with 100 % survival rate.*

Key words: *Multiple shoots, Nodal explants, Menispermaceae, Tinospora formanii*

I. Introduction

The genus *Tinospora* within the family Menispermaceae is reputed for its medicinal properties [1]. *Tinospora* species have been reported to have prominent roles in the traditional medicinal practices of Australia, Africa and Asia. Some medicinally important species includes *T. cordifolia, T. malabarica, T. tementosa, T. crispa, T. uliginosa* etc. [2]. *Tinospora* species has been identified as one of the most commercially exploited plants in pharmaceuticals by the task force on conservation and sustainable use of medicinal plants in India. The estimated annual demand of this species used in the preparation of crude herbal drugs in the Indian system of medicines is 10,000 tonnes [3]. It is therefore important to to select, multiply and conserve genetically superior planting material of important medicinal plants for assured uniformity and desired quality. Owing to poor seed set and poor germination in natural conditions and overexploitation of the species, conventional propagation has a limited scope for improvement of *Tinospora*. *In vitro* regeneration can play an important role in selecting, multiplying and conserving the critical genotypes of this species. *Tinospora formanii* Udayan & Pradeep, a new species of the family Menispermaceae is a woody dioecious climber reported from the Western Ghats of Thrissur district, Kerala, South India. *Tinospora formanii* is endemic to Kerala growing at an altitude of 500–650 m. *Tinospora formani* is allied to *T. smilacina* Benth. with smooth, shining, papery bark peeling off into scales and prominent leaf-scars. Leaves are alternate, ovate to elliptic-lanceolate, coriaceous, and glabrous with reticulation more prominent on lower surface. Female inflorescence is greenish-yellow, glabrous, stout compound elongated pseudoracemes with six petals. Drupes are globose and red when mature [4].

II. Materials And Methods

2.1 Collection and preparation of explants

Nodal explants collected from one year old vegetatively propagated disease free plants were cultured on MS media for callus initiation. The explants were first washed in running tap water for 30 minutes and then soaked in distilled water for 30 minutes. The explants were then treated with Tween 20 emulsifier solution (0. 01% v/v) for five minutes. After distilled water wash for 2–3 times, the explants were taken to the laminar air flow chamber for further sterilization. The nodal explants were initially sterilized in ethyl alcohol (70 %) for 30 seconds followed by 0.1 % mercuric chloride for five minutes. The treated explants were then washed four times with sterile distilled water to make them free from sterilants. Finally using sterile forceps, explants were transferred to sterile Petri dishes

and the nodes were cut into vertical sections of 1.0–1.5 cm length with sterile scalpel and inoculated into the media.

2.2 Media and culture conditions

The nodes were then cultured on semi solid MS medium [5] supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. The pH of the media was adjusted and maintained to 5.8 by using 0.1 N NaOH or 0.1 N HCl after the addition of growth regulators. The medium was autoclaved at 121°C, 15 psi pressure for 15 min. All these cultures were incubated in growth room at 25 ± 2°C under 16 hour photoperiod at a relative humidity of 60–70 percent with a light intensity of 3000 lux provided by cool white fluorescent lamps.

2.3 Multiple shoots induction and elongation

MS medium supplemented with BAP (0.5, 1.0, 1.5, 2.0 mg L^{-1}) either alone or in combination with IBA (0.2, 0.5 mg L^{-1}) was used to study the effect of their initial establishment on multiple shoot induction. MS without growth regulators served as control. Subcultures were done every 14 days interval into fresh medium. Thirty days after inoculation, the multiple shoots initiated were separated. Small clumps of 1-2 shoots were again inoculated into fresh induction medium for further multiplication. During this period, the number of shoots proliferated for each of the treatment was recorded. Two weeks after sub culturing the lengthy well grown shoots with 2-3 leaves were transferred into the elongation media. For elongation MS medium supplemented with BAP (0.5, 0.75, 1.0, 1.5, 2.0 mg L^{-1}) along with IBA (0.5 mg L^{-1}) was used. During the elongation process, the shoot length and number of leaves produced were recorded to assess the best treatment.

2.4 *In vitro* rooting and hardening

Thirty days after subculturing in the shoot elongation media, the elongated micro shoots were transferred to the rooting media. Full strength and half strength MS medium as basal and with IBA were used for *in vitro* rooting. The parameters recorded for *in vitro* rooting was number of roots, number of days for rooting and root length. The individual rooted plants (20 days old) were taken out, washed free of agar and transferred to Soilrite mix^{TC}: garden soil mixture (1:1), hardened in rectangular box in growth room for 15 days and later transferred to mist chamber for further establishment.

2.5 Statistical Analysis

The experiments were carried out in completely randomized block design and repeated three times each with 20 replications for each of the treatment. The observations were tabulated and statistical analysis was carried out, as per [6] and the results were interpreted.

III. Results

The effect of BAP on multiple shoot induction was studied by culturing shoot tip explants in MS medium supplemented with BAP alone at four different concentrations (0.5, 1.0, 1.5, 2.0 mg L^{-1}). None of the explants differentiated into multiple shoots even after 30 days of culture. Invariably the explants showed browning and eventually died. This shows that BAP alone was detrimental for explants. With BAP (0.5, 1.0, 1.5, 2.0 mg L^{-1}) in combination with two levels of IBA (0.2 and 0.5 mg L^{-1}), 2-3 multiple shoots developed within 6-19 days. High concentration of IBA 0.5 mg L^{-1} with BAP showed 90% shoot induction (Table 1). Higher concentration of BAP with higher concentration of IBA induced multiple shoots within 6-14 days whereas lower concentration of IBA with BAP delayed the shoot induction. The multiplication was more than two times when the concentration of IBA was increased from 0.2 to 0.5mg L^{-1} with high concentrations of BAP (1.5 and 2.0 mg L^{-1}). Highest number of shoots per explants (12.75) was produced on MS medium containing 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} IBA which was the single best treatment for shoot proliferation (Fig.1; Table 2).

To induce shoot elongation, the individual shoots were excised and inoculated in MS medium containing BAP at five levels (0.5, 0.75, 1.0, 1.5, 2.0 mg L^{-1}) in combination with 0.5 mg L^{-1} IBA. Shoot length of 4.30 cm was recorded after 30 days of sub culturing in 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} IBA which was comparable with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} IBA (4.16 cm). The shoot length did not further increase with lower concentrations of BAP. Maximum number of 4.25 leaves on an average was produced in high concentration of BAP 2.0 mg L^{-1} with 0.5 mg L^{-1} IBA. The number of leaves obtained with 1.5 mg L^{-1} BAP was 4 with same concentration of IBA (0.5 mg L^{-1}) which is at par with the above treatment. The leaves were broad and green in colour in medium containing BAP 1.5 and 2.0 mg L^{-1} with 0.5 mg L^{-1} IBA. The leaves were narrow and dull green in colour with BAP 1.0 mg L^{-1} with 0.5 mg L^{-1} IBA (Table 3). The number of leaves reduced with lower concentrations of BAP (0.5, 0.75 mg L^{-1}). Half strength MS basal medium was found to be the optimum medium for rooting. Strong healthy roots of 3.18 cm length initiated within 8 days in this medium and were favourable for transfer during hardening without any damage. Full strength MS medium produced thin long roots of 3.88 cm length in 14 days which did not survive after hardening. The roots initiated in half strength

MS with IBA 2.0 mg L^{-1} were thick with a root length of 1.87 cm and did not support successful establishment after hardening (Table 4).

Table 1: Effect of BAP and IBA on multiple shoot induction using nodal explants

Treatments	MS + Plant growth regulators (mg L^{-1})	% explants showing shoot formation	Number of days for shoot induction
T_0	MS basal (control)	0.0 ± 0^{g}	0.00^{f}
T_1	BAP 0.5 + IBA 0.2	40.0 ± 2.88^{f}	19.3 ± 1.15^{de}
T_2	BAP 1.0 + IBA 0.2	53.3 ± 5.77^{de}	18.3 ± 1.00^{d}
T_3	BAP 1.5 + IBA 0.2	65.0 ± 5.77^{cd}	18.6 ± 1.53^{d}
T_4	BAP 2.0 + IBA 0.2	73.3 ± 3.33^{c}	15.3 ± 0.57^{c}
T_5	BAP 0.5 + IBA 0.5	78.0 ± 1.66^{c}	14.0 ± 0.57^{c}
T_6	BAP 1.0 + IBA 0.5	90.3 ± 2.35^{ab}	10.6 ± 1.15^{b}
T_7	BAP 1.5 + IBA 0.5	93.0 ± 4.30^{a}	7.6 ± 0.57^{a}
T_8	BAP 2.0 + IBA 0.5	95.0 ± 5.00^{a}	6.3 ± 0.50^{a}

Values are means ± S.E. of three independent experiments, each consisted of 20 replicates per treatment. Treatment means followed by same letter within column are not significantly different from each other at P= 0.05; comparison by Duncan's Multiple Range Test.

Table 2: Effect of BAP and IBA on shoot proliferation after 30 days of culture

Treatments	MS + Plant growth regulators (mg L^{-1})	No of shoots / explant
T_0	MS basal (control)	0.00^{g}
T_1	BAP 0.5 + IBA 0.2	1.66 ± 0.33^{f}
T_2	BAP 1.0 + IBA 0.2	3.00 ± 0.82^{e}
T_3	BAP 1.5 + IBA 0.2	5.00 ± 0.57^{de}
T_4	BAP 2.0 + IBA 0.2	6.00 ± 1.15^{de}
T_5	BAP 0.5 + IBA 0.5	4.25 ± 0.95^{d}
T_6	BAP 1.0 + IBA 0.5	8.50 ± 0.57^{bc}
T_7	BAP 1.5 + IBA 0.5	10.50 ± 0.57^{b}
T_8	BAP 2.0 + IBA 0.5	12.75 ± 0.88^{a}

Values are means ± S.E. of three independent experiments, each consisted of 20 replicates per treatment. Treatment means followed by same letter within column are not significantly different from each other at P= 0.05; comparison by Duncan's Multiple Range Test.

Table 3: Effect of BAP and IBA on shoot length and number of leaves

Treatments	MS+ Plant growth regulators (mg L^{-1})	Shoot length (cm)	No: of leaves
T_0	MS basal (control)	0.00^{d}	0.00^{d}
T_1	BAP 0.50 + IBA 0.5	2.20 ± 0.30^{c}	2.25 ± 0.25^{c}
T_2	BAP 0.75 + IBA 0.5	2.80 ± 0.45^{b}	2.50 ± 0.28^{c}
T_3	BAP 1.00 + IBA 0.5	3.37 ± 0.60^{b}	3.50 ± 0.57^{ab}
T_4	BAP 1.50 + IBA 0.5	4.30 ± 0.52^{a}	4.00 ± 0.57^{a}
T_5	BAP 2.00 + IBA 0.5	4.16 ± 0.31^{a}	4.25 ± 0.25^{a}

Values are means ± S.E. of three independent experiments, each consisted of 20 replicates per treatment. Treatment means followed by same letter within column are not significantly different from each other at P= 0.05; comparison by Duncan's Multiple Range Test.

Table 4: Effect of media and plant growth regulators on *in vitro* rooting

Treatments	Media strength	Plant growth regulators and additives(mg L^{-1})	Number of roots	No: of days for rooting	Root length (cm)
T_0	Full strength MS	Basal	2.25 ± 0.50^{b}	16.5 ± 0.75^{c}	3.88 ± 0.27^{a}
T_1		IBA 2.0	2.50 ± 0.28^{b}	12.7 ± 0.47^{b}	2.1 ± 0.31^{b}
T_2	Half strength MS	Basal	4.50 ± 0.50^{a}	8.0 ± 0.28^{a}	3.18 ± 0.51^{a}
T_3		IBA 2.0	3.25 ± 0.25^{a}	7.3 ± 0.48^{a}	1.87 ± 0.49^{c}

Values are means ± S.E. of three independent experiments, each consisted of 20 replicates per treatment. Treatment means followed by same letter within column are not significantly different from each other at P= 0.05; comparison by Duncan's Multiple Range Test.

Fig. 1. Micropropagation of *Tinospora formanii*. **A,** Habit of *Tinospora formanii*; **B &C,** Initiation of shoot bud and multiple shoot induction from nodal segments in MS supplemented with BAP 1.5 mg/L+ IBA 0.5 mg/L; **D, E & F**, Shoot bud initiation and multiple shoot induction in MS supplemeted with BAP 2.0 mg/L + IBA 0.5mg/L; **G,**Shoot elongation in MS supplemented with BAP 1.5 mg/L + IBA 0.5 mg/L; **H,** Rooting in half MS basal; **I&J,** Hardened *in vitro* regenerated plants of *Tinospora formanii* after acclamatisation.

IV. Discussions

The different concentrations of BAP and IBA showed significant variations on percent response of explants (%) and on number of shoots per explants. Multiple shoot induction was obtained from nodal explants on MS medium supplemented with varying concentrations of BAP with IBA 0.1 and 0.2 mg L^{-1}. Callus induction and organogenesis has been reported from cultured vegetative parts such as stem, leaf and nodal explants from an elite *in vivo* grown mature plant of *Tinospora cordifolia* on MS medium supplemented with different hormonal concentrations [7]. *In vitro* propagation in *T. cordifolia* has been reported from nodal segments through axillary shoot proliferation [8, 9]. Media composition plays a key role in morphogenesis as nutritional requirement for optimal growth of a tissue under *in vitro* conditions varies with species [10]. In the present study, the use of BAP alone as growth regulator did not result in bud initiation. Incorporation of IBA with BAP was found to be stimulatory for shoot multiplication of *T. formanii*. Among the cytokinins tested, N

benzyladenine (BA) has been found to be more effective than Kinetin for axillary shoot proliferation in *T. cordifolia* [8]. BAP combined with auxins exhibit synergistic effect and hence has also been used by number of researchers [11]. Relatively high levels of auxins are required to promote the growth of axillary shoots and reduce apical dominance. Cytokinin growth regulators added to shoot culture media to promote axillary shoot growth, usually inhibit root formation. Single shoots or shoot clusters should therefore be moved to a different media for *in vitro* rooting [12]. In the present study good response for rooting was observed with half strength MS medium than full strength MS medium. The time taken in MS basal media was found to be twice as that in half MS. Strong healthy roots were produced in a week in half MS basal medium. Similar results have been reported in *T. cordifolia* where microshoots rooted spontaneously in half strength than full strength basal medium [8].

V. Conclusion

The protocol can be successfully applied for the mass multiplication and ex situ conservation of this valuable threatened taxon. Also the tissue culture raised plants can be used for pharmacological studies, thus avoiding the need to collect them from natural environment.

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