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IN VITRO PROPAGATION OF *FIBRAUREA DARSHANI* UDAYAN AND RAVIKUMAR- AN ENDEMIC AND ENDANGERED PLANT FROM WESTERN GHATS

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ABSTRACT
Fibraurea darshani belonging to the family Menispermaceae is an endangered plant endemic to Western Ghats. A protocol was developed for *in vitro* regeneration and multiplication of *Fibraurea darshani* through the culture of mature nodal explants. Sterilization treatment with alcohol 70% for 30 seconds followed by 0.1% Mercuric chloride ($HgCl_2$) for 8 minutes showed high asepsis and survival rate. The maximum shoot induction from nodal segments was observed on Murashige and Skoog (MS) medium containing 3% sucrose and 0.8% agar supplemented with 2.0 mg L^{-1} of 6-Benzylaminopurine (BAP). 93% of the explants initiated 1-2 buds in 9-10 days of culture. Shoot proliferation resulted from newly formed shoot clumps which were cultured on fresh medium with the same concentration of growth regulators as that of the induction medium. An average of 5.6 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 Kinetin (KN) 0.1 mg L^{-1}. The best medium for rooting was found to be half MS medium supplemented with IBA 1.0 mg L^{-1}. Three to four strong healthy roots initiated in 7 days. The regenerated plants with well developed shoots and roots after hardening were successfully established in net house with 83 % survival rate.

KEY WORDS: *Fibraurea darshani*, Menispermaceae, Nodal explants, Multiple shoots.

INTRODUCTION

The family Menispermaceae consists of about 70 genera and 450 species that are found in tropical lowland regions. The plants belonging to this family are generally climbing or twining shrubs. Leaves are alternate or lobed, flowers small cymose and seeds are usually hooked or reniform. The family is a rich source of alkaloid and terpenes (Sharma *et al*., 2010). The family is well known as an important source of isoquinoline alkaloids, one of the largest groups of natural products which display interesting pharmacological activity (Shamma, 1972). The genus *Fibraurea* Lour. belonging to the family Menispermaceae with its two species namely *F. tinctoria* Lour. and *F. recisa* Pierre. is mostly restricted to East and South East Asia including Andaman and Nicobar islands (Forman, 1985; Kubitzki *et al*., 1993, Pramanik, 1993; Mabberely, 2005).

F. tinctoria Lour., have been used in traditional medicines of Thailand and some other Asian countries (Perry and Metzger, 1980). A decoction of the roots and stems is used to treat dysentery, diabetes, eye diseases and stomach conditions. The plant has diuretic, analgesic and sedative activity (Siwon *et al*., 1981). *F. tinctoria* has been reported to exhibit antimicrobial activity (Pongpan *et al*., 1982; Avirutnant and Pongpan, 1983; Grosvenor *et al*., 1995). Methanol extracts of the stems of *F. tinctoria* was reported to have antioxidant activity on DPPH radical, and showed cytotoxic activity against brine shrimp and human cancer cell line MCF-7, breast adenocarcinoma (Keawpradub *et al*., 2005).

The stem of *F. recisa* is commonly used for the treatment of various skin diseases by the minority Yao, Zhuang and Miao in Yunnan Province of China as an ethno-remedy. From the stem and root bark of *F. tinctoria* the alkaloids magnoflorine, pseudocolumbamine, dehydrocorydalmine and palmatrubine were isolated and identified by their spectral data. By means of TLC the alkaloids berberine and berberrubine were found to be present in minute amounts (Siwon *et al*., 1981). The stem of *F. recisa* is an effective antifungal herb, and its major active component was reported to be alkaloidal compounds. Bioassay tests revealed that the water-soluble berberines are the most important antifungal substances (Rao *et al*., 2009).

A relatively new species, *F. darshani* Udayan & Ravikumar has been reported from Karnataka and Kerala states of South India. *F. darshani* is a woody dioecious climber found in semi ever green forests. Flowering and fruiting is observed in January-April months. *F. darshani* is an endangered plant endemic to Western Ghats (Udayan *et al*., 2007). So there is an immediate need to mass propagate these plants as a measure for conservation. *In vitro* propagation allows mass multiplication of plants under aseptic conditions from limited sources with high degree of genetic uniformity in the progeny. Here we report an efficient protocol for micro propagation of *F. darshani* using nodal segments as explants. To our

knowledge, this is the first report on *in vitro* study of *F. darshani.*

MATERIALS & METHODS

Collection of explants

The stem cuttings of the plant, *F. darshani* were collected from Vellanipacha forests of Western Ghats, Thrissur district, Kerala and authenticated by taxonomist Dr P. S. Udayan. The stem cuttings were vegetatively propagated in pots and maintained at Botanical Garden of Sree Krishna College, Guruvayur. Nodal segments collected from these one year old vegetatively propagated disease free plants served as explants.

Surface sterilization

The nodal segments of 2-3 cm length excised from the plant were first washed in running tap water for 30 minutes and then soaked in distilled water for 30 minutes to remove dirt and dust. The explants were then treated with Tween 20 emulsifier solution 0. 01% (v/v) for five minutes. The explants were then taken to the laminar air flow chamber for further sterilization after distilled water wash for 2–3 times. The nodal explants were initially sterilized in ethyl alcohol 70 % (v/v) for 30 seconds followed by Mercuric chloride 0.1 % (w/v) for eight minutes and subsequently washed four-five times with sterile distilled water to remove traces of sterilants. Finally the explants were transferred to sterile Petri dishes using sterile forceps and the nodes were cut into vertical sections of 1.0–1.5 cm length with sterile scalpel and inoculated into the MS media.

Culture media

The nodes were cultured on semi solid MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. All the salts used were of analytical grade. 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 20 minutes.

Culture conditions

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 and the relative humidity was maintained between 50-60%.

Sub culturing

Sub culturing was done at regular intervals. The shoots induced were transferred to fresh semi solid media with growth regulators at an interval of 10-12 days.

Multiple shoot induction and elongation

Single nodal explants were vertically inoculated in each test tube containing MS medium supplemented with different combinations of auxins, BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}) was used to study the effect of their effect on multiple shoot induction. MS medium without growth regulators served as control. The multiple shoots initiated were separated thirty days after inoculation. Small clumps of 1-2 shoots were again inoculated into fresh medium for further multiplication. The percentage explant showing shoot formation, number of days for shoot induction and the number of shoots proliferated for each of the treatment was recorded during this period. Two weeks after sub culturing, 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 KN (0.1 mg L^{-1}) was used. During the elongation process, the shoot length and number of leaves produced were recorded to assess the best treatment.

In vitro rooting and hardening

The elongated micro shoots were transferred to the rooting media thirty days after sub culturing in the shoot elongation media. Half strength MS medium as basal and with IBA 0.25, 0.5, 1.0, 1.5 mg L^{-1} was used to study the effect on 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 in autoclaved distilled water and transferred to small plastic pots with Soilrite: garden soil mixture (1:1), hardened in rectangular box in growth room for 15 days. Plants were then transferred to larger earthen pots (18 cm diameter) containing garden soil: compost (1:1) and kept in green house conditions. The number of surviving plants was recorded periodically.

Experimental Design and Statistical Analysis

The experiments were carried out in completely randomized block design and repeated three times each with 12 replications for each of the treatment. The observations were tabulated and all the data recorded were subjected to analysis of variance (ANOVA) and the significant differences among means were compared by Duncan Multiple Range Test (DMRT) at 5 % level of significance (IBM SPSS ver. 19) and the results were interpreted (Harter,1960). All the results were expressed in Mean ± Standard error.

RESULTS & DISCUSSION

The effect of auxins on multiple shoot induction and proliferation was studied by culturing nodal explants on MS medium supplemented with different concentrations of BAP at six different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}). The shoots were multiplied by subculturing of the proliferated shoots and repeated transfer of mother explants with the shoots. Generally the usage of BAP is considered as most suitable for promoting large-scale multiplication and micropropagation of various plant species (Shrivastava and Banerjee, 2008). Highest percentage of multiple shoot induction (93.3%) and number of shoots per explant (5.60 ±0.14) was produced on MS medium containing 2.0 mg L^{-1} BAP which was the single best treatment for shoot proliferation (Table 1; Fig.1). Similar findings have been reported by Raghu *et al*., 2006 in *Tinospora cordifolia* of the family Menispermaceae where cytokinin BA has found to be more effective than KN for shoot proliferation. The effectiveness of BA to induce multiple shoots was also reported in woody species such as *Pterocarpus marsupium* (Anis *et al*., 2005), *Cleome spinosa* (Albarello *et al*., 2006), *Balanites aegyptiaca* (Siddique and Anis, 2009) and *Psidium guajava* (Rai *et al*., 2009). The promotory effect of BA over other cytokinins could be due to its easy permeability, increased affinity for active cell uptake, less resistance to the enzyme cytokinin oxidase, or receptor abundance in its perception apparatus which interacts with the coupling elements in the signal transduction chain (Burch and Stuchbury, 1987). The second best treatment for shoot induction was found to be in MS medium

containing 1.5 mg L^{-1} BAP where 76% of explants induced shoots. But the number of days for shoot formation was found to be increased (13.0 ±0.16) in MS containing 1.5 mg L^{-1} BAP when compared to 2.0 mg L^{-1} BAP (10.3 ±0.16). At both lower and higher concentration of BAP (0.5, 1.0, 2.5 and 3.0 mg L^{-1}), the percentage of shoot formation and the number of shoots formed was considerably reduced (Table 1 & 2). Reduction in the number of shoots in the concentration higher than optimal level has also been reported for several woody plants (Rai *et al*., 2009).

TABLE 1: Effect of BAP 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	30.0 ± 0.83^{f}	20.3 ± 0.33^{e}
T_2	BAP 1.0	43.3 ± 0.47^{e}	19.3 ± 0.29^{e}
T_3	BAP 1.5	76.0 ± 0.47^{b}	13.0 ± 0.16^{b}
T_4	BAP 2.0	93.3 ± 0.96^{a}	10.3 ± 0.16^{a}
T_5	BAP 2.5	66.0 ± 1.66^{c}	14.6 ± 0.33^{c}
T_6	BAP 3.0	60.3 ± 0.67^{d}	15.6 ± 0.44^{cd}

Values are means ± S.E. of three independent experiments, each consisted of 12 replicates per treatment. Treatment means followed by same letter within column are not significantly different from each other at $P \leq 0.05$; comparison by Duncan's Multiple Range Test.

TABLE 2: Effect of BAP 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^{f}
T_1	BAP 0.5	1.20 ± 0.10^{e}
T_2	BAP 1.0	1.66 ± 0.09^{d}
T_3	BAP 1.5	3.00 ± 0.15^{bc}
T_4	BAP 2.0	5.60 ± 0.14^{a}
T_5	BAP 2.5	4.00 ± 0.17^{b}
T_6	BAP 3.0	3.50 ± 0.19^{b}

Values are means ± S.E. of three independent experiments, each consisted of 12 replicates per treatment. Treatment means followed by same letter within column are not significantly different from each other at $P \leq 0.05$; comparison by Duncan's Multiple Range Test.

TABLE 3: Effect of BAP and KN 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^{e}	0.00^{d}
T_1	BAP 0.50 + KN 0.1	2.42 ± 0.06^{d}	1.75 ± 0.14^{c}
T_2	BAP 0.75 + KN 0.1	2.72 ± 0.04^{d}	2.00 ± 0.21^{c}
T_3	BAP 1.00 + KN 0.1	3.95 ± 0.03^{b}	2.67 ± 0.14^{b}
T_4	BAP 1.50 + KN 0.1	4.60 ± 0.04^{a}	4.25 ± 0.16^{a}
T_5	BAP 2.00 + KN 0.1	3.35 ± 0.09^{c}	2.94 ± 0.16^{b}

Values are means ± S.E. of three independent experiments, each consisted of 12 replicates per treatment. Treatment means followed by same letter within column are not significantly different from each other at $P \leq 0.05$; comparison by Duncan's Multiple Range Test.

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 0.1 mg L^{-1} KN to induce shoot elongation. Shoot length was recorded after 30 days of sub culturing. The shoots elongated to a maximum length of 4.6 ± 0.04 cm in MS supplemented with 1.5 mg L^{-1} BAP and 0.1 mg L^{-1} KN. Maximum number of leaves (4.25 ± 0.16) was also produced in MS medium containing BAP 1.5 mg L^{-1} with 0.1 mg L^{-1} KN. Previous studies have reported the synergistic effect of BAP and KN on shoot elongation in other crops (Shirin and Rana, 2007; Saha *et al*., 2007). Similar effects of lower concentrations of cytokinin on shoot elongation have been reported by Kaur *et al*. (1998), Dave and Purohit (2002), Arya *et al*. (2008) and Gayathri *et al*. (2009). The shoot length or the number of leaves did not further increase with higher concentrations of BAP at 2.0 mg L^{-1} with 0.1 mg L^{-1} KN (Table 3).

Elongated shoots were excised from parent pant and transferred to half strength MS basal medium with or without growth regulators. Root initiation from microshoots was delayed in the medium without auxin. The percentage of shoots forming roots and the root length per shoot significantly varied with different concentrations

of IBA. Half strength MS basal medium with IBA 1.0 mg L^{-1} was found to be the optimum medium for rooting (95.3%). Strong healthy roots of 3.36 ±0.22 cm length initiated within 7-8 days in this medium and were favourable for transfer during hardening without any damage. 95% of microshoots rooted in this medium (Table 4). Thin long brittle roots were produced (4.42 ±0.15 cm) when the IBA concentration was increased to 1.5 mg L^{-1} and the percentage of rooting was also found to be significantly reduced (74.60 %). The supplementation of auxin either singly or in combination for rooting has been reported in many plant species (Gopi *et al*., 2006; Baksha *et al*., 2007; Kalidass and Mohan, 2009). Earlier studies have reported that the addition of IBA favours rooting in some medicinal plants (Chandra *et al*., 2006; Sivanesan, 2007; Meena *et al*., 2010). IBA has been shown to be very effective in root induction in various species of tropical trees such as *Azadirachta indica* (Chaturvedi *et al*., 2004), *Albizia odoratissima* (Rajeswari and Paliwal, 2008), *Psidium guajava* (Rai *et al*., 2009). IBA is the most commonly used auxin to stimulate the rooting process in shoots because of its its weak toxicity, high ability to promote root initiation (Weisman *et al*., 1988) and great stability in comparison to naphthalene acetic acid and indole-3-acetic acid (Zolman *et al*., 2000).

The individual rooted plants (20 days old) were taken out, washed free of agar and transferred to vermiculite: garden soil mixture (1:1), hardened in rectangular box in growth room for 15 days and later transferred to green house for further establishment. The regenerated plants were uniform morphologically and exhibited similar growth characteristics and vegetative morphology to the mother plants.

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

Values are means ± S.E. of three independent experiments, each consisted of 12 replicates per treatment. Treatment means followed by

Media strength	Plant growth regulators and additives (mg L^{-1})	Rooting (%)	Number of roots	No: of days for rooting	Root length (cm)
Half strength MS	Basal	$31.33 \pm 1.1.5^{e}$	1.30 ± 0.16^{d}	13.40 ± 0.54^{d}	0.98 ± 0.25^{e}
	IBA 0.25	41.60 ± 1.52^{d}	1.75 ± 0.14^{d}	11.80 ± 0.83^{c}	1.68 ± 0.17^{d}
	IBA 0.5	65.00 ± 1.00^{c}	2.56 ± 0.18^{bc}	10.60 ± 0.70^{b}	2.25 ± 0.21^{c}
	IBA 1.0	95.30 ± 0.57^{a}	5.50 ± 0.16^{a}	7.25 ± 0.50^{a}	3.36 ± 0.22^{b}
	IBA 1.5	74.60 ± 1.50^{b}	3.25 ± 0.14^{b}	8.00 ± 0.81^{a}	4.42 ± 0.15^{a}

same letter within column are not significantly different from each other at $P \leq 0.05$; comparison by Duncan's Multiple Range Test.

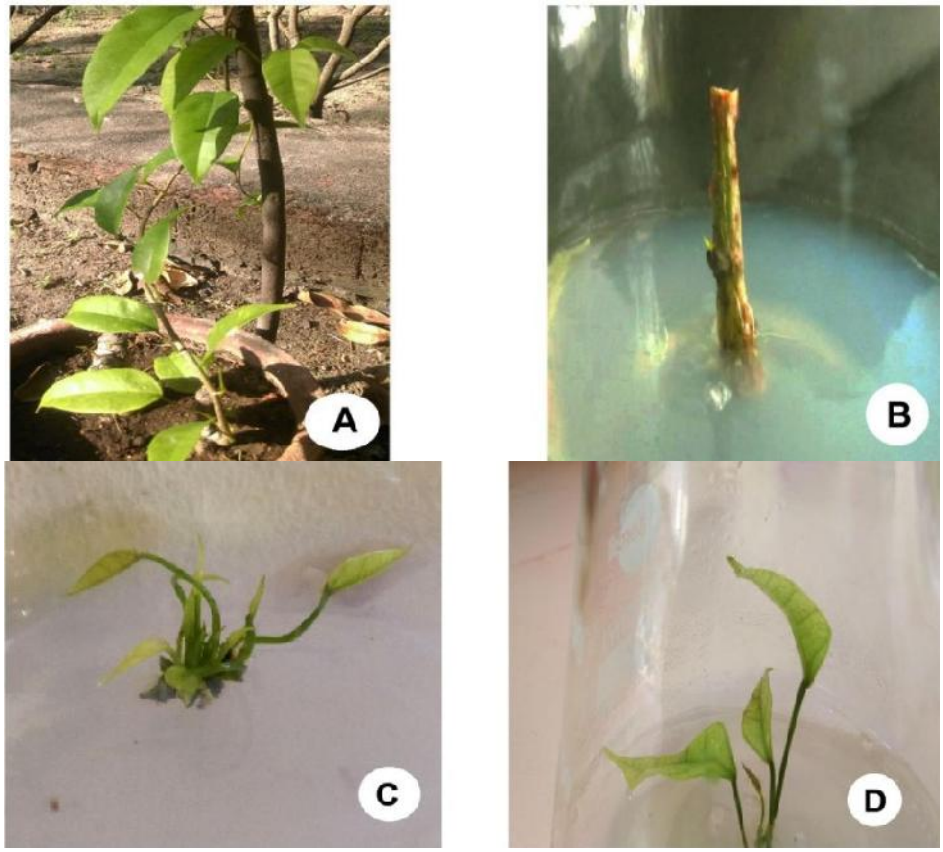

FIGURE 1: Micropropagation of *Fibraurea darshani* A, Habit of *Fibraurea darshani;* B, Initiation of shoot bud from nodal segments; C, Multiple shoot induction from nodal segments in MS supplemented with BAP 2.0 mg/L; D, Shoot elongation in MS supplemented with BAP 1.5 mg/L + KN 0.1 mg/L; E, Rooting in half MS basal + IBA 1.0 mg/L; F, Hardened *in vitro* regenerated plants of *Fibraurea darshani* after acclimatization.

CONCLUSION

In this study it can be concluded that BAP promoted shoot induction and multiplication whereas BAP with KN synergistically enhanced shoot elongation in *F. darshani*. The protocol developed through this study offers a quick and reliable method for the direct and large-scale multiplication of this endangered and endemic plant that would find application in *ex situ* conservation.

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