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In vitro regeneration, callus induction and rhizogenesis in *Ficus krishnae*: A rare endangered plant

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Ficus krishnae which belongs to the family Moraceae is an endangered plant species with medicinal potential. Due to over-exploitative commercial use, it has become endangered hence demands immediate conservation. Application of *in vitro* strategies in conservation of plants is an efficient technique to produce true-to-type plantlets. In the present study, nodal segment explants were used as induction of shoots on Murashige & Skoog (MS) medium fortified with various concentrations of benzylaminopurine (BAP) in combination with 0.5 mg/l naphthalene acetic acid (NAA) to determine optimum levels of BAP and NAA for multiple shoot formation. Cytokinins, BAP and kinetin (Kn) were tested for shoot regeneration from nodal and axillary shoot tip explants. Among all the treatments BAP and Kn tested BAP at 4.0 mg/l and at 2.5 mg/l responds optimum in shoot regeneration from nodal explants and axillary shoot tip, respectively. Average number of sprouted shoots per explant and length of these shoots were determined after three weeks of explant inoculation. Axillary-shoot tip explants show best results of callus induction at 2 mg/l 2,4 diphenoxy acetic acid (2, 4-D) with a response percentage of 83.33%. Moreover, it was also noted that among the different concentrations of indole butyric acid (IBA) tested, IBA at the concentration of 1.5 mg/l resulted best in inducing the maximum number (12.66 ± 0.33) of *in vitro* adventitious roots from the callus. In this study combination of BAP + NAA (4.0 + 0.5) mg/l and 2 mg/l (2,4-D) resulted better in shoot induction and callus formation respectively in *F. Krishnae*.

Keywords: Endangered plant; *Ficus krishnae*; BAP; 2,4-D; callus

Introduction

Krishndona *(Ficus krishnae)* is mainly found in India, tropical Africa and Sri Lanka. The unique feature of the tree is that its leaves have a pocket-like fold at the base[1]. *F. krishnae* is treated as sacred tree in India due to its peculiar nature of cup forming leaves. Indian folklores are associated with the species regarding the formation of cone shaped leaves also called 'Makkhan Katori' (butter cup). It has been used in number of folklore medicines. Various plant parts are used to treat ulcers, vomiting, fever, inflammation and leprosy. Latex is aphrodisiac, and is used in treatments of piles and gonorrhea[2]. Additionally, it has been proven to have anti-diabetic and anti-hyper lipidemic activity[3]. Phenolic compounds and a halogenated coumarin in 2H-chromen-2-one named 3-chloro-7-methoxy-4-methyl-chromen-2-one has been isolated from this plant[4]. As a result of high commercial exploitation for a long time, the natural reserve of this plant has been dwindling alarmingly. As a consequence, it is included under rare and endangered category[5-6].

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Application of *in vitro* strategies in conservation and management of important plant species has been given priority when genetic resources are getting depleted rapidly from natural flora. *In vitro* conservation provides numerous advantages over conventional propagation methods like mass production of true-to-type and disease free plants of elite species in speedy manner irrespective of the season requiring smaller space and tissue source. Thus, it provides a reliable technique for *in vitro* conservation of various rare, endangered threatened germplasm[7]. Besides, it facilitates distribution and easy exchange of the elite plant germplasm[8]. The present study undertakes to develop an efficient method for shoot induction, shoot regeneration, and callus induction from nodal and axillary shoot-tip explants for the large scale multiplication of *F. krishnae* plantlets as very little work is reported on tissue culture of *F. krishnae* so far.

Materials and Methods

Plant Material, Surface Sterilization and Culture Condition

The plant materials viz., actively growing nodal segments and axillary shoot-tips were collected from

the Botanical Garden, St. John's College, Agra, India. The selected explants were free from any visible sign of diseases. The explants were cut with a scissor and kept in a conical flask containing double distilled (DD) water and brought to the laboratory. The explants were washed in running tap water for about 30 minutes. Then a few drops of liquid detergent Tween 20 was put into the flask and the explants were agitated in the detergent solution for a minute and poured off. The explants were again washed in running tap water for 30 minutes. Then 100 ml of water was added to the flask and 200 mg bavistin, 50 mg streptomycin was added and the flask was vigorously agitated. The explants were kept in the disinfection solution for 3 - 3.5 hours. The disinfection solution was poured off and the explants were washed three times in DD water and then decontaminated in a 3.5% sodium hypochlorite solution for 15 min. The explants were then washed with sterile distilled water. Aseptic axillary shoot-tip (10 mm in length) and nodal segment explants were cultured on MS medium[9] supplemented with 30 g l^{-1} sucrose, 8 g l^{-1} agar and different plant growth regulators (PGRs) alone or in combination for *in vitro* shoot induction and shoot regeneration. The concentration and combinations of PGR's tested are indicated in Table 1-3. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH and/or 0.1 N HCl before gelling with 8 g l^{-1} agar and autoclaved at 121°C for 20 min. The cultures were maintained at 25 ± 2°C under a 16-h photoperiod with 50 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by cool white fluorescent tubes (40 W, Philips, India) with 55 ± 5% of relative humidity.

Effects of PGRs

Axillary shoot-tips and nodal segments explants were cultured on MS medium fortified with various concentrations of 2, 4-D (ranged from 0.5 to 4.0 mg/l). The MS media supplemented with different concentrations of 2, 4-D produced variable degree of callus induction and proliferation from different explants used in the present study. Among the nutrient media (MS) used, the MS medium along with the selected hormonal concentrations alone was found suitable for maximum callus induction and proliferation as compared to other hormonal media concentrations and hence incorporated in the further experiment. Regenerating cultures were cut into pieces and sub-cultured onto the optimized combination of PGR's dispensed in the flasks and the data were recorded. Sub-culturing was done within 4 or 5 weeks of interval as required. After the sub-culturing

Table 1 — Effect of BAP, NAA and IBA on shoot and root induction

S. No.	Medium	Hormonal conc. (mg/l)	Responded explants number	%	No. of shoots per explants	Mean no. of roots/callus	Mean root length in cm
1	BAP + NAA	2.5 + 0.5	1	8.33	1.25 ± 0.25	-	-
2	BAP + NAA	3.0 + 0.5	4	33.33	2.00 ± 0.40	-	-
3	BAP + NAA	3.5 + 0.5	6	50.00	2.25 ± 0.25	-	-
4	BAP + NAA	4.0 + 0.5	10	83.33	3.00 ± 0.40	-	-
5	BAP + NAA	4.5 + 0.5	8	66.66	2.50 ± 0.50	-	-
6	IBA	0.5	-	-	-	2.33 ± 0.88	0.76 ± 0.12
7	IBA	1.0	-	-	-	5.33 ± 0.88	1.26 ± 0.29
8	IBA	1.5	-	-	-	12.66 ± 0.33	3.13 ± 0.18

Table 2 — Effect of cytokinins BAP and Kn on shoot regeneration

S. No.	Cytokinin	Conc. (mg/l)	Nodal segment		Axillary shoot-tip explants	
			Average number of shoot	Shoot length	Average number of shoots per explant	Mean shoot length
1	BAP	1.0	-	-	0.50 ± 0.28	0.33 ± 0.07
2	BAP	2.0	0.75 ± 0.25	0.33 ± 0.08	0.75 ± 0.25	0.63 ± 0.15
3	BAP	2.5	-	-	2.00 ± 0.40	1.23 ± 0.11
4	BAP	3.0	1.50 ± 0.28	0.43 ± 0.08	-	-
5	BAP	4.0	2.00 ± 0.40	1.36 ± 0.08	-	-
6	Kn	0.5	-	-	0.25 ± 0.25	0.26 ± 0.02
7	Kn	1.5	1.00 ± 0.40	0.43 ± 0.06	0.50 ± 0.28	0.93 ± 0.19
8	Kn	2.5	1.25 ± 0.25	0.66 ± 0.16	-	-
9	Kn	3.0	1.50 ± 0.28	1.06 ± 0.18	1.00 ± 0.40	1.46 ± 0.21

Table 3 — Effect of concentration of 2, 4-D in MS basal medium on callus induction from axillary shoot tip and nodal explant of *Ficus krishnae*

S. No.	Conc. of 2,4 D (mg/l)	Axillary shoot tip					Nodal explant			
		No. of tubes inoculated	No. of tubes responded	Rate of callus induced (%)	Days to callus	Degree of callus	No. of tubes responded.	Rate of callus induced (%)	Days to callus	Degree of callus
1	1.0	12	3	25.00	48	+	-	-	-	-
2	1.5	12	5	41.66	43	+ +	-	-	-	-
3	2.0	12	10	83.33	28	+ + +	1	08.33	51	+
4	2.5	12	7	58.33	35	+ +	2	16.66	48	+
5	3.0	12	4	33.33	41	+	5	41.66	39	+ +
6	3.5	12	-	-	-	-	7	58.33	37	+ +
7	4.0	12	-	-	-	-	9	75.00	30	+ + +
8	4.5	12	-	-	-	-	6	50.00	40	+ +

the callus was transferred on fresh prepared MS medium. The MS medium was solidified with 8 g/1 agar and was fortified with different concentration of auxin IBA (0.5, 1.0 and 1.5 mg/l). Activated charcoal was also added in the same medium. After 14 days callus was transferred to the new medium and the callus showed induction of adventitious rooting, and the induced roots showed elongation on the same medium. There are five replicates of each treatment.

Statistical Analysis

Data was recorded throughout the study and was subjected to statistical analysis. Mean values and respective standard errors were calculated. The data was represented in terms of average number of shoots per explants and percentage of callus induction after 2-3 weeks of inoculation. Similarly in other parameters (shoot regeneration and formation of adventitious rooting from explants), data analysis was carried out computationally as mean ± standard error (SE).

Results and Discussion

Effect of BAP with NAA on Shoot Induction

The nodal segments were inoculated on MS medium supplemented with different concentrations of BAP (2.5, 3.0, 3.5, 4.0 and 4.5 mg/l) and 0.5 mg/l NAA. After 3 weeks, (Fig 1C) multiple shoots emerged from the explants. The maximum number (3.0 ± 0.40) shoots were obtained at 4.0 mg/l BAP and 0.5 mg/l NAA, (Table 1). On increasing or decreasing the optimum (4.0 mg/l) BAP concentrations percentage response and mean number of shoots per explant were reduced. The minimum shoot number was observed at 2.5 mg/l BAP with 0.5 mg/l NAA (Fig. 1B). However, nodal segments of *F. krishnae* failed to induce multiple shoot buds on PGR-free basal MS medium (control). Therefore, it was mandatory to augment the culture medium with cytokinin alone or in combination with auxin to induce multiple shoot buds. Cytokinins were reported to play a key role in DNA synthesis and cell division, which might be the reason for induction of multiple shoots[10]. Bud sprouted after 10-15 day of culture (Fig. 1B). Similar results were evaluated in *Ficus carica*[11], in which multiple shoots emerged from shoot tip explants on MS medium fortified with different concentrations of BAP within 4 weeks. It was also observed that maximum shoot induction (62.50%) were observed at BAP 2 mg/l and NAA 1 mg/l in *Prosopis cineraria*[12] and *Calotropis prosera*[13]. Moreover, Munshi[14] reported that when MS medium supplemented with 1.0 BA + 0.1 NAA (mg/l) induces the maximum number of shoots from nodal explants in *Ficus benghalensis* L. The current study also supported that higher levels of BAP induces the shooting from the explants.

Effect of BAP and Kn on Shoot Regeneration from Nodal Segment Explants and Axillary Shoot-Tip Explants

BAP and Kn were tested in different concentrations for shoot regeneration from nodal segment explant of *F. Krishnae*. Among them BAP at 4 mg/l resulted best with maximum number of mean shoots (2.0 ± 0.40) and average number of shoot length (1.36 ± 0.08) (Table 2, Fig. 1D), while Kn in different concentration tested, 3.0 mg/l was observed best for shoot regeneration in 5 weeks with mean number of shoots (1.50 ± 0.28) and (1.06 ± 0.18) mean shoot length. Axillary shoot-tip explants were also accessed for the shoot regenerative potentialities. It is reported that the shoot tips cultured on PGR-free MS basal medium (control) failed to initiate multiple shoot buds even after 4 weeks of incubation. However, the supplementation in different concentrations of cytokinins (BAP 1.0, 2.0 and 2.5 mg/l and Kn 0.5, 1.5 and 3 mg/l) resulted in shoot regeneration with

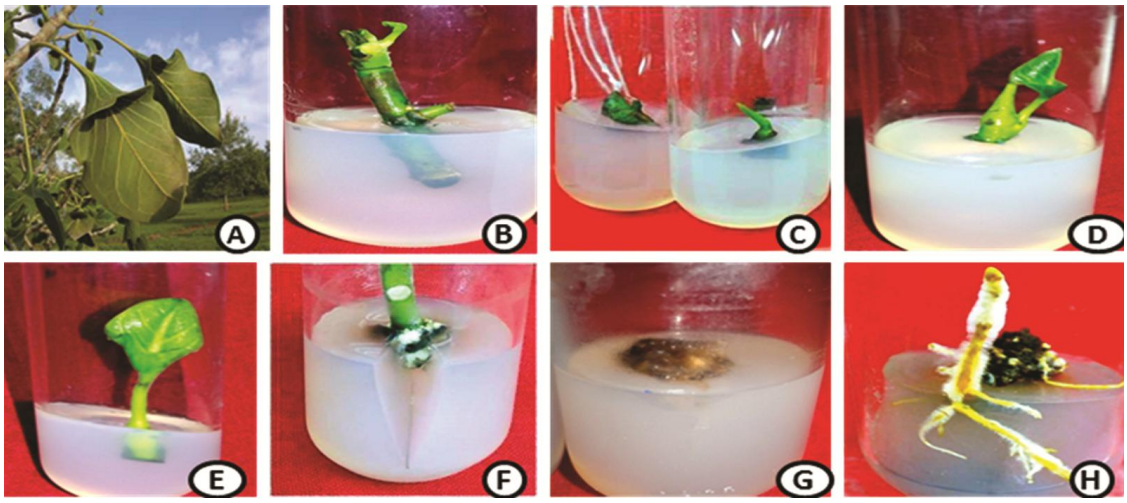

Fig. 1 — (A) A mature plant of *Ficus krishnae*, (B) Axillary sprouted bud after 10 days of inoculation, (C) BAP + NAA induced shoot regeneration after 3 weeks of culture, (D) BAP + Kn induced shoot regeneration after 5 weeks of culture, (E) BAP + Kn induced shoot tip culture after one week of culture, (F) Callus induction from shoot tip explants after 10-12 days of culture, (G) Callus development after 3 week of culture and (H) Root induction from callus obtained 5 weeks of culture.

the formation of new sets of leaves within 8-10 days of incubation (Fig 1E). Adenine-based cytokinins BAP and Kn tested, BAP (4.0 mg/l) was found to be the best as inducing a mean number of 2.00 ± 0.40 shoots per explant. Kn shows optimum results on shoot induction at 3 mg/l with average number of shoots 1.50 ± 0.28, but the mean shoot number and shoot induction was less in comparison to BAP (Table 2).

The superiority of BAP over other cytokinins with respect to multiple shoot bud induction through shoot-tip explants has also been documented for several medicinal and aromatic plant species such as *Stevia rebaudiana*[15-16]. The role of BAP and Kn in shoot regeneration is also elucidated[17]. The promoting effect of BAP over other cytokinins could be due to its easy permeability, increased affinity for active cell uptake, less resistance to the cytokinin oxidase or receptor abundance in its perception apparatus which interacts with the coupling elements in the signal transduction chain. Other reasons for superiority of BAP may be attributed to the ability of plant tissues to metabolize BAP more readily than other synthetic PGRs or to the ability of BAP to induce production of natural hormones such as zeatin within the tissue[18].

Callus Induction from Axillary Shoot-Tip Explant and Nodal Segment Explant

Axillary shoot-tip explants and nodal segment explants of *F. krishnae* were tried for callus induction. In the current study axillary shoot-tip explant was more responsive than nodal segment explant. After sterilization the axillary shoot-tip explant were inoculated on 2, 4-D containing MS medium to induce callus (Fig. 1F). In control set MS medium was devoid of 2, 4-D and failed to induce callus. Different concentration of 2, 4-D was tested (1, 1.5, 2, 2.5, and 3 mg/l). Optimum callusing response of axillary shoot-tip explants was noticed after 10 to 12 days on 2 mg/l of 2, 4-D (Fig 1F) with a response percentage of 83.33. With further increase or decrease the concentration of 2, 4-D, the percentage of callus response of explants was decreased and took long time for callus induction than optimum concentration. Callus was brownish green in color and compact in texture (Table 3). Different concentrations of 2, 4-D (2.0, 2.5, 3.0, 4.0 and 4.5 mg/l) were also tested to induce callogenesis from nodal segment explants of *F. krishnae* (Fig. 1G). Two cm young healthy nodal explants were inoculated on 2, 4-D containing MS media. Optimum callogenesis was noticed on 4 mg/l 2, 4-D containing MS medium which became visible after 9-10 days of inoculation. The percentage of explant was 75 at the 4 mg/l 2, 4-D, and at concentration 3.5 mg/l and 4.5 mg/l the response percentage of explant was 58 and 50 respectively. On increasing or decreasing 2, 4-D concentration beyond an optimal level callogenesis was reduced respectively. The callus was greenish brown in color and at some portions was green. Obtained callus was compact in texture after 3 weeks of culture (Table 3, Fig 1G). Our results are in accordance with earlier findings of several workers, such as, one study revealed that 3.5 mg/l 2, 4-D was appropriate for callus induction

in *Triticum aestivum*[19]. Ather *et al*[20] also evaluated that 100% callus were produced from shoot apices of *Saccharum officinarum* L. by addition of 2, 4-D in the concentration of 3.0 mg/l with MS medium.

Effect of IBA on Direct *In Vitro* Adventitious Rooting from Callus

The proliferated callus obtained from nodal segment explant of *F. krishnae* was compact in texture. The color of callus was greenish brown and some portions were green in color where the callus was actively dividing, after two sub-culturing the callus was transferred on fresh prepared MS medium. The MS medium was solidified with 8 g/l agar, and was fortified with different concentration of auxin IBA (0.5, 1.0 and 1.5 mg/l). It is well studied that exogenously applied natural or synthetic auxins favor rooting[21]. Activated charcoal was also added in the same medium. After 14 days callus was transferred to MS medium supplemented with IBA and the callus showed the induction of rooting and the induced roots showed elongation in the same medium after 5 weeks (Fig. 1H). Among the different concentration tested IBA, the concentration of 1.5 mg/l resulted best in inducing the maximum number of *in vitro* adventitious roots from the callus. At this concentration (1.5 mg/l) the average number of induced roots from callus was observed 12.66 ± 0.33 with the average root length in cm 3.13 ± 0.18, and at 0.5 mg/l IBA the callus showed minimum average number of roots in cm 2.33 ± 0.88 with root length 0.76 ± 0.12 (Table 1). Similar results have been evaluated in various studies for example, direct *in vitro* rooting are reported from explant derived callus of *Erythrina variegata* on MS medium supplemented with IBA and other growth regulators[22]. In an another report direct root organogenesis was obtained when the MS was enriched with IBA (0.5 and 1.5 mg/l)[23]. Bagadekar and Jayaraj[24] also evaluated that frequent rhizogenesis was obtained from the leaf callus and stem callus of *Heliotropium indicum*. It is also found that addition of activated charcoal (AC) with IBA (2 mg/l) exhibited maximum root formation in *Bacopa monnieri*. Teshome and Feyissa[25] studied that the maximum root number (14.10 ± 1.47) and root length (1.01 ± 0.10 cm) in *Glinus lotoides* were obtained on a medium supplemented with 1.5 mg/l indole-3-butyric acid (IBA).

Conclusion

It is concluded that nodal segment explant shows optimum response for shoot induction on MS medium fortified with 4 mg/l BAP in combination of 0.5 mg/l NAA. In *F. krishnae* it was noticed that axillary shoot-tip explants were more responsive for shoot regeneration and callus formation than nodal segments. For culture initiation, MS medium with BAP 2.5 mg/l in axillary shoot-tip explant was found optimum while Kn was less effective than BAP growth hormone 2, 4-D can be used for the callus culture of *F. krishnae*, among different concentrations but 2 mg/l 2, 4-D was optimum concentration for callus induction of axillary shoot-tip explant and 4 mg/l 2, 4-D was optimum for nodal explants. Indole butyric acid (1.5 mg/l) results best adventitious *in vitro* rooting of *F. krishnae*. Tree *F. Krishnae* is an endangered plant; hence this study will serve as a milestone for standardization to develop the protocol of optimization for mass multiplication and rooting for future research.

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