In vitro culture of *Feronia limonia* (L.) Swingle from hypocotyl and internodal explants

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Abstract

The hypocotyl and internodal segments from *in vitro* grown seedlings of *Feronia limonia* (L.) Swingle (wood apple) were cultivated on Murashige and Skoog's (1962, MS) medium supplemented with N^6-benzyladenine (BA) or adenine (ADE) or kinetin (KN) at 0.5 to 5 μM. The optimum response was recorded on the medium containing 2 μM BA. An average of 12 and 8 shoots were developed from hypocotyl and internodal explants, respectively, after eight weeks of culture. The shoots were excised, and the residual explants were transferred to fresh medium where again they developed shoots. Up to three such passages resulted in the production of shoots from repeatedly subcultured explants and an average of 24 - 36 shoots per explant was obtained. The *in vitro* developed shoots produced roots when transferred to half strength MS medium supplemented with 1 μM 1-naphthaleneacetic acid (NAA). The developed plantlets were successfully transferred to mixture of soil, sand and coco-peat (1:1:1) and hardened in controlled environment. Hardened plants were transplanted to soil in greenhouse.

Additional key words: adventitious shoots, medicinal plant, micropropagation, organogenesis.

Introduction

The *Feronia limonia* (L.) Swingle (*Rutaceae*) is fruit yielding tree, common in the dry plains of India and Sri Lanka and frequently grown throughout Southeast Asia. Conventional propagation of *Feronia limonia* can be achieved from seed, which results in a high degree of genetic and phenotypic variation. Micropropagation can provide an opportunity to obtain large number of homogenous plants. In case of *Feronia limonia* attempts at propagation have so far been restricted to shoot formation from nodal explant (Purohit and Tak 1992), cotyledon explant (Hossain *et al.* 1994), and leaf explants (Hiregoudar *et al.* 2003). Other tissues of *Feronia limonia* have not yet been reported as initial explants for *in vitro* culture. In the present paper we describe a simple protocol for the rapid and recurrent propagation of *Feronia limonia* through high frequency adventitious shoot regeneration from hypocotyl and internodal explants derived from axenic seedlings.

Materials and methods

Fruits of *Feronia limonia* were collected from a tree grown in the Botanical Garden, Karnatak University, Dharwad, India. Seeds were removed from the fruits, washed with running water for 10-15 min, immersed in an aqueous solution of liquid detergent (1 % *Labolin*, *Ranbaxy Fine Chemicals*, Mumbai, India) for 5 min and washed in 1 % sodium hypochlorite for 15 min and later with 0.2 % mercuric chloride for 10 min followed by three to four rinses in sterile distilled water. The surface disinfected seeds were transferred to glass tubes (20 cm^3,

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Abbreviations: ADE - adenine; BA - N^6-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; KN - kinetin; MS - Murashige and Skoog; NAA - 1-naphthaleneacetic acid.

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Borosil Glasswares, Mumbai, India) plugged with cotton containing basal MS medium with 2 % sucrose and 0.8 % agar (*Himedia*, Mumbai, India). Hypocotyl and internodal segments (5 - 6 mm) were dissected from the 15-d-old axenic plants and were cultured horizontally into glass tubes containing MS medium.

Medium was prepared using MS salts, vitamins, 2 % sucrose and this basal medium used as control. Plant growth regulators tested included various cytokinins like N^6-benzyladenine (BA), adenine (ADE) or kinetin (KN) at concentrations of 0.5, 1.0, 2.0, or 5.0 μM. The medium pH was adjusted to 5.7 prior to addition of agar and autoclaved at 120 °C for 20 min. Cultures were incubated at 24 ± 2 °C under 16-h photoperiod using cool, white fluorescent light irradiance of 40 μmol m^{-2} s^{-1}. Explants were sub-cultured to fresh medium of the same composition at an interval of every four weeks.

Shoots of 3 - 5 cm in length were cultured on to full, half and quarter strength MS supplemented with 2 % sucrose medium and MS medium with 2 % sucrose and growth regulators like 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) at 0.5 and 1.0 μM concentrations were tested for rooting.

Well-developed plantlets were removed from culture vessels and washed with sterilized distilled water to remove the traces of medium. These plantlets were transplanted to plastic cups containing a mixture of autoclaved soil and sand (1:1), soil, sand and vermiculite (1:1:1) and soil, sand and coco-peat (1:1:1) and were maintained in growth chambers wherein temperature was 24 ± 2 °C, relative humidity 80 %, irradiance of 100 μmol m^{-2} s^{-1} for 16-h photoperiod. The plants were irrigated with Hoagland solution once in a week. After eight weeks the hardened plants were transferred to pots containing soil and farmyard manure (1:1) and maintained in greenhouse.

Cultured explants were periodically collected at different intervals and were fixed in FAA (formalin, glacial acetic acid, 40 % ethanol, 10:5:85) for 12 h at room temperature, dehydrated through a ethanol-butyl alcohol series and embedded in paraffin wax. The tissues sectioned at a thickness of 7 μm were stained with 0.05 % toluidine-blue solution (Fowke and Rennie 1996) and examined under compound microscope (*Nikon*, Tokyo, Japan).

A completely randomized design was used for all treatments. One hypocotyl/internodal explant was cultured per test tube and there were twelve replicates for each treatment/experiment. The experiments were repeated twice. The cultures were observed periodically and morphological changes were recorded at weekly intervals. Percentage of responding explants and number of shoots developed per responding explants were recorded. Results were subjected to analysis of variance (*ANOVA*) and mean values were separated according to Duncan's multiple range test at $P = 0.05$ level.

Results and discussion

The hypocotyl segments on primary culture medium supplemented with BA, KN and ADE remained green and showed enlargement during first week in culture and developed small nodular protuberances in second week. Further these protuberances developed into adventitious shoots within 5 - 6 weeks. Explants cultured on MS basal medium alone failed to induce such response. After 8 weeks of culture on medium with 1.0 μM BA, an average of 5.05 shoots formed per explant. Maximum of 12.48 shoots developed from the explants on medium supplemented with 2.0 μM BA (Fig. 1*A*). Callus was also developed from the cut end of hypocotyl explants but it did not affect the growth of shoot buds. Histological investigation revealed that adventitious shoots were developed directly from competent cells of the explants (Fig. 1*B*). KN (0.5 - 5.0 μM) supplemented media also induced development of adventitious shoots (Table 1) from the hypocotyl explants and optimum 10.22 shoots were developed on medium supplemented with 2.0 μM KN. Among these cytokinins tested, BA was better in shoot induction than KN and ADE.

The internodal explants, which were cultured on medium supplemented with BA, also showed similar response like that of hypocotyl explants and developed adventitious shoots in 5 - 6 weeks. Explants cultured on MS basal medium and MS medium supplemented with

Table 1. Effect of cytokinins supplemented to MS medium on hypocotyl and internodal explants of *Feronia limonia* (L.) Swingle. Means followed by the same letter are not significantly different according to DMRT at $P = 0.05$.

Cyt.	Conc. [μM]	Responding explants [%]		Mean number of shoots	
		hypocotyl	internode	hypocotyl	internode
BA	0.5	100.00	79.16	1.33 f	3.87 c
	1.0	94.44	75.00	5.05 d	4.62 b
	2.0	94.44	79.16	12.48 a	8.37 a
	5.0	100.00	83.33	8.99 c	3.87 c
KN	0.5	100.00	0.00	3.55 c	0.00 d
	1.0	61.11	0.00	0.60 fg	0.00 d
	2.0	94.44	0.00	10.22 b	0.00 d
	5.0	100.00	0.00	4.49 d	0.00 d
ADE	0.5	16.66	0.00	0.16 g	0.00 d
	1.0	16.66	0.00	0.16 g	0.00 d
	2.0	16.66	0.00	0.16 g	0.00 d
	5.0	20.83	0.00	0.21 g	0.00 d

KN and ADE neither showed appreciable enlargement nor initiated shoot buds although they remained green up to 5 - 6 weeks. On medium supplemented with 1.0 µM BA, internodal explants induced 4.62 shoots, while explants cultured on medium supplemented with 2.0 µM BA induced optimum of 8.37 shoots per explant (Table 1). Addition of higher concentration (5.0 µM) in MS medium was not beneficial in producing more adventitious shoots compared to 2.0 µM BA. Internodal explants showed development of callus formation in cut ends of explants but callus development did not hinder the development of shoots. Histological study of responsive internodal explants showed the direct development of shoot primordia from the competent cells.

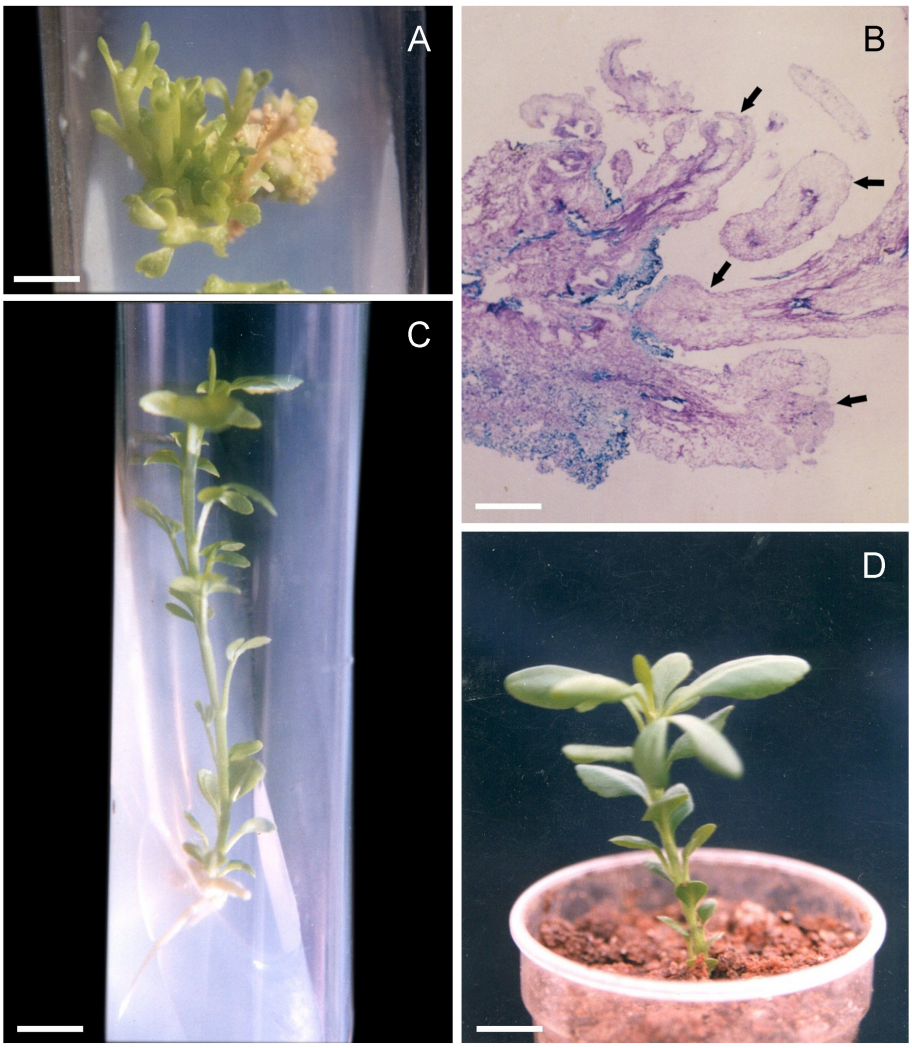

Fig. 1. *A* - Six-week-old culture showing multiple shoots on MS medium supplemented with 2.0 µM BA (*bar* = 0.45 cm). *B* - Longitudinal section of hypocotyl showing direct development of shoots (*bar* = 0.66 cm). *C* - Rooting of shoot on quarter strength MS medium supplemented with 1.0 µM NAA (*bar* = 1.31 cm). *D* - Plantlet transferred to pot containing mixture of soil, sand and coco peat (1:1:1) (*bar* = 1.60 cm).

Cytokinins especially BA was necessary for shoot induction in *Feronia limonia* explants lacking pre-existing meristem. Effectiveness of BA to induce adventitious meristem organogenesis has been well documented in some other plant species, example *Citrus aurantifolia* and *C. reticulata* (Perez *et al*. 1997), cumin (Tawfik and Noga 2001). Similarly BA was found to be most effective cytokinin during *in vitro* regeneration of other woody species *Madhuca longifolia* (Rout and Das 1993), *Cornus florida* (Kaveriappa *et al*. 1997), *Syzygium cuminii* (Jain and Babbar 2000), *Amorpha fruticosa* (Gao *et al.* 2004). The hypocotyl and internodal explants developed adventitious shoots from competent cells and callus from non-competent cells, thus it is suggested here that in *Feronia limonia*, tissues simultaneously underwent two types of response to the medium supplemented with cytokinin in induction medium. One of these responses, the proliferation of callus like tissue

from the non-competent cells and the other was the initiation of new adventitious shoot buds from competent cells. Similar observation was made with the *in vitro* cultured hypocotyl and internodal explants of cumin (Tawfik and Noga 2001). It is noticeable that the formed callus like tissues did not initiate new shoot buds during the prolonged incubation up to six weeks of culture on induction medium containing BA or KN or ADE. Hiregoudar *et al*. (2003) reported the adventitious shoot development from leaf explants on MS medium supplemented with BA and thus addition of BA alone is enough for the induction of adventitious shoots from the seedling explants of *Feronia limonia*. However, Hossain *et al*. (1994) have used combination of cytokinins (BA and KN) for the induction of adventitious shoots from the cotyledons of *Feronia limonia* and reported that BA favored direct shoot organogenesis, whereas KN favored callogenesis. In the present studies it is observed that irrespective of cytokinins used the explants involved in two kinds of responses, *i.e*., direct development of shoots from competent cells of explants and callogenesis from non-competent cells. Shoot induction in *Feronia limonia* in the present study was best at a considerably low concentration of BA (2.0 μM). The low concentrations of plant growth regulators may reduce the chance of inducing somaclonal variations (Skirvin *et al*. 1994).

Shoots developed on hypocotyl and internodal explants were excised and the original explants were transferred to shoot multiplication medium (MS + 2.0 μM BA) and were maintained for 8 weeks. On the residual explants, new adventitious shoots were developed again and were also excised at the end of culture period. Residual explants were once again transferred to fresh shoot induction medium, on which adventitious shoots developed again. The explants so transferred continued to produce shoots up to third passage. During the fourth passage the adventitious shoot production was scanty and explants turned brown. Adopting this procedure of shoot excision and re-culturing of original explant, an average of 24 - 36 shoots could be obtained per explant. Similar approach of re-culturing of explants to fresh shoot induction medium was adopted earlier for other plant species (Kaveriappa *et al*. 1997, Jain and Babbar 2000, Martin 2004).

The shoots raised *in vitro* were transferred to full, half and quarter strength MS medium supplemented with NAA or IAA or IBA (0.5 or 1.0 μM) for the induction of roots (Table 2). Shoots cultured on full strength MS medium supplemented with 0.5 μM IBA induced 3.0 roots per shoot after four weeks of culture. Half strength MS medium supplemented with IBA (0.5 μM) also induced roots but frequency of roots decreased to 1.83. Roots developed on IBA supplemented medium invariably developed callus at their apical ends. 4.33 roots were developed per shoot on full strength medium supplemented with 1.0 μM NAA and optimum 7.16 roots developed per shoot on half strength MS medium supplemented with 1.0 μM NAA. Roots developed on medium supplemented with NAA were healthy and did not develop callus. IAA (1.0 μM) supplemented medium also developed roots but frequency was very low (Table 2). No roots were observed from regenerated shoots maintained on full, half and quarter salt strength MS medium free of growth regulators. Frequency and number of roots developed were more in half strength medium supplemented with NAA (1.0 μM) and similar positive effect of low salt media on rhizogenesis has been reported earlier in *Cercis canadensis* (Mackey *et al*. 1995) *Sterculia urens* (Purohit and Dave 1996) and *Syzygium cuminii* (Jain and Babbar 2000). As observed in other medicinal woody taxa, *Picrorhiza kurroa* (Lal *et al*. 1988), *Saussurea lappa* (Arora and Bhojwani 1989) NAA at 1.0 μM was found superior to IBA or IAA for rooting of shoots.

Table 2. Effect of auxins supplemented to full strength, half strength and quarter strength MS medium on *in vitro* rooting of shoots of *Feronia limonia* (L.) Swingle. Means followed by the same letter are not significantly different according to DMRT at $P = 0.05$.

MS medium	Auxins	[μM]	Shoots rooted [%]	Mean number of roots
1	NAA	0.5	83.33	0.83 g
1	NAA	1.0	83.33	4.33 b
1	IAA	1.0	83.33	0.83 g
1	IBA	0.5	50.00	3.00 d
1	IBA	1.0	66.66	1.33 f
1/2	NAA	1.0	83.33	7.16 a
1/2	IBA	0.5	33.33	1.83 e
1/4	NAA	0.5	83.33	0.83 g
1/4	NAA	1.0	100.00	3.33 c
1/4	IAA	1.0	33.33	0.33 h

Plants (3 - 5 cm height) with 6 - 8 expanded leaves and well-developed roots were transplanted to plastic cups containing mixture of soil, sand and vermiculite (1:1:1), mixture of soil, sand and coco-peat (1:1:1) and mixture of soil and sand (1:1) and reared in controlled conditions. Of the three potting media tested, mixture of soil, sand and coco-peat was found suitable for growth of plants (Fig. 1*D*) and 66.6 % of plantlets survived on this media after 8 weeks of transplantation. After eight weeks, plants were transferred to mixture of soil and farmyard manure (1:1) and maintained in greenhouse.

This study shows that shoot buds can be successfully regenerated on *Feronia limonia* hypocotyl and internodal explants through direct organogenesis. By using this protocol, a number of plants of *Feronia limonia* can be produced at any time of the year, as this tree species is a good candidate for afforestation program.

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