

## Full Length Research Paper

# ***In vitro* callus induction and plantlet regeneration of Indian red wood, *Soymida febrifuga* A. Juss (Roxb.)**

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*In vitro* seedling explants of *Soymida febrifuga* were screened for their callus induction and morphogenic potential by culturing on Murashige and Skoog (MS) medium supplemented with naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), indole3acetic acid (IAA) alone or in combination with benzyladenine (BA), kinetin (KN), 2-iP (2-isopentynyl adenine), coconut milk. Multiple shoots were regenerated from cotyledonary node, shoot tip explants derived callus by one stage process where caulogenesis occurred on the primary callus. However cotyledons, leaves and root segments gave rise to adventitious shoots from callus by two stage method of indirect organogenesis. High frequency of shoot differentiation was observed from cotyledon and leaf on MS medium fortified with BA (8.9 to 13.3  $\mu$ M) in combination with NAA (0.54  $\mu$ M) compared to other explants. Further, when elongated shoots were subjected to rooting, we observed shoot necrosis consistent to our previous findings. Similarly, we could overcome this problem by supplementing calcium to MS rooting medium. *In vitro* rooted plantlets were successfully transferred to soil, exhibiting a normal development. Altogether, our protocol provides a rapid technique for mass multiplication as well for *ex situ* conservation of this endemic medicinal plant.

**Key words:** Seedling, cotyledon, leaf, shoot necrosis, rooting.

## INTRODUCTION

*Soymida febrifuga* A. Juss (Roxb.) is a folk endemic medicinal tree indigenous to India (Anonymous, 1952; Chopra et al., 1956). The decoction of the bark has bitter resin used in vaginal infections, rheumatic pains and stomach pains. It is also used for the treatment of wounds, dental diseases, uterine bleeding, haemorrhage and as anticancer agent (Ambaye et al., 1971). Bark

extracts are acrid, refrigerant, anti-helmintic, possess aphrodisiac and laxative properties, good for sore throat, cough and asthma, remove vata and cure tridosha fevers (Yoganasimhan, 1996). The bark is crushed and mixed with water and administered in cough. Besides removing blood impurities, various parts of the plant are treated for ulcers, leprosy, dysentery and have anti-inflammatory

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activity. The bark is employed in intermittent fevers and general debility, in advanced stages of dysentery and diarrhoea. It is as good as the cinchona bark in treating malaria.

Decoction of the bark is used in Yunani medicine for curing fevers, bowels and a good substitute for oak-bark used for vaginal infections, preparation of gargles and enemata. The bark is a bitter tonic and a dilution of 1 in 20 is given in one ounce doses three times a day for malarial fever. Decoction of bark is said to be useful for the treatment of gum infection, tongue sores and for fixing loose teeth (Kirtikar and Basu, 2003). Root bark is extensively used in treating leucorrhoea, menorrhagia, dysmenorrhoea. In traditional medicine, the stem bark is widely used for the treatment of a variety of diseases including wounds, asthma, rheumatism and cancer.

Macropropagation of *S. febrifuga* through stem cuttings is cumbersome that restricts propagation due to difficulty in rooting. Seeds have low germination capacity and seedlings are prone to insect attack. Hence the micropropagation by shoot organogenesis from callus cultures of *S. febrifuga* has been implemented in the present study, an effective method for large scale production of medicinal plants. Adventitious shoots under *in vitro* conditions can arise indirectly in two ways: (a) on primary callus produced on the original explant (b) on callus in which a capacity for morphogenesis has been induced, but which shows no organ formation until it is excised and transferred to another medium. The potential genetic variability associated callus culture is important for genetic improvement and selection strategies, which provides a continuous and constant year round production of plant material. Successful *in vitro* plant regeneration of *S. febrifuga* by direct organogenesis has been reported by our group (Chiruvella et al., 2013). The current study reports our novel attempts to establish micropropagation using callus cultures from juvenile seedlings.

## MATERIALS AND METHODS

### *In vitro* seed germination

Seeds were surface sterilized with 0.05% HgCl<sub>2</sub> for 15 min followed by 3 to 4 rinses in sterilized distilled water. Seeds were germinated in MS medium containing sucrose under aseptic conditions (Chiruvella et al., 2013).

### Culture medium and conditions

Seedling explants are in general more responsive in proliferation of callus than explants derived from mature trees (Chiruvella et al., 2013). Explants such as cotyledons, cotyledonary node, leaves, nodes, shoot tip and root segments were dissected from 15-day-old aseptic seedlings and inoculated on MS medium supplemented with auxins [indole3acetic acid (IAA), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D)] and cytokinins (benzyladenine (BA), coconut milk (CM), 2-isopentynyl adenine (2-iP) and kinetin) alone or in combination. The nature and morphology of

the callus was observed and the callus was then transferred to MS medium variously supplemented with high concentrations of cytokinins alone and in combination with low concentrations of auxins for the shoot induction and proliferation of multiple shoots. Natural growth additives and organic supplements like coconut milk and casein hydrolysate were also incorporated into the medium to study their influence in shoot organogenesis.

The media were congealed with agar (0.8%), and sucrose 3% was used as a source of carbohydrate. The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 15 min. All cultures were incubated at 25 ± 2°C under 16 h photoperiod with light supplemented by cool white fluorescent tubes (25 μ moles m<sup>-2</sup> s<sup>-1</sup>). Each treatment consisted of seven replicates and experimental unit was one explant per vessel. Sub-culturing was done by transferring the micro-shoots to fresh shoot induction medium.

### *In vitro* rooting and acclimatization

Elongated shoots were dissected from proliferated shoot cultures and rooting was carried out on MS full and half strength medium with and without calcium supply containing IBA and IAA either individually or in combination (Chiruvella et al., 2013). Rooted plantlets (after 6 to 8 weeks) were removed from culture tubes, washed thoroughly and were transplanted to pots containing a mixture of soil, peat moss and soilrite.

### Data analysis

Each culture tube with one shoot explants was considered as one replicate. Each treatment in each set of experiments consists of 20 replicates and each experiment was repeated three times. Standard error of means was calculated in each experiment. The data was statistically analyzed using one way analysis of variance (ANOVA) and means were compared using the Duncan's multiple range test (DMRT) at the 0.05% level of significance

## RESULTS

In the present study, we regenerated shoots from aseptic seedling explants such as cotyledonary node, shoot tip, cotyledons and leaves derived callus by one stage or two stage method of indirect organogenesis on MS medium fortified with various auxins (NAA, 2,4-D and IAA) alone or in combination with cytokinins [BA, KN, 2-iP and traditional Chinese medicine (TCM)]. Callus was induced in all the explants, although the nature of the response varied with the explant source as well as with the concentration of different auxins and cytokinins used. The varied response among the explants for callus initiation may be attributed to the differences in their physiological status as stated by (Murashige, 1974; Nagarathna et al., 1991). The data on the nature and color of the callus formed from various explants in the presence of different cytokinins concentrations with MS medium was compiled in Table. 1. MS medium responded better and is selected for callus induction as well as caulogenesis. The age of the explant was found to be very important in terms of facilitating the formation of callus capable of continued proliferation and plant regeneration (Bandopadhyay et al., 1999). In an experiment involving the use of explants

**Table 1.** Effect of different plant growth regulators on callus induction from seedling explants of *S. febrifuga*.

PGR ( $\mu\text{M}$ )				Nature of response and morphogenic ability						
BA	NAA	2,4-D	2-ip	Cotyledon		Leaves		Root		
0.89	-	-	-	Light green fragile	+++	Yellowish green compact	+	Light yellowish friable compact nodular		+
0.89	1.07	-	-	Light cream soft	-	Light cream friable	++	Light yellowish friable compact nodular with green patches		++
0.89	2.69	-	-	Light cream soft	-	Light cream friable	++	Light brown soft friable		-
2.22	-	-	-	Light green friable	+++	Light green compact nodular	+++	Light brown soft compact nodular		+
2.22	1.07	-	-	Dark green compact nodular	+++	Dark green compact nodular	+++	Light brown friable nodular		+++
2.22	2.69	-	-	Light cream soft	+	Light green compact nodular	++	Light brown compact nodular		++
4.4	-	-	-	Light green friable	++	Light green compact nodular	++	Brown compact nodular		++
4.4	1.07	-	-	Dark green compact nodular	+++	Dark green compact nodular	+++	Brown compact nodular		+
4.4	2.69	-	-	Dark green compact nodular	+++	Dark green compact nodular	+++	Brown compact nodular friable		++
-	1.07	-	-	Light yellowish cream soft	-	Light creamish compact loose friable	++	Dark brown loose friable		+
-	2.69	-	-	Light yellowish cream soft	-	Light creamish compact	++	Dark brown loose friable		-
-	-	2.26	-	Brown compact friable	-	Brown compact	-	Dark brown loose friable		-
-	-	2.26	15	NR	-	NR	-	Hard,dark brown compact		-
-	-	2.26	30	NR	-	NR	-	Hard,dark brown compact		-
-	-	4.52	-	Dark brown compact friable	-	Brown compact	-	Dark brown loose friable		-
-	-	4.52	15	NR	-	NR	-	Hard,hark brown loose friable		-
-	-	4.52	30	NR	-	NR	-	Hard,dark brown compact		-
-	-	-	15	Green Soft friable	++	Light green compact	++	Light yellowish brown compact nodular		++
-	-	-	30	Light yellowish-green friable	+	Yellowish green compact	+	Light brown compact nodular		+
2.22	-	2.26	-	Brown hard compact	-	Brown loose friable	-	Dark brown loose friable		-
2.22	-	4.5	-	Brown hard compact	-	Brown loose friable	-	Dark brown loose friable		-
2.22	-	-	15	NR	-	NR	-	Dark brown loose friable		-
2.22	-	-	30	NR	-	NR	-	Dark brown loose friable		-

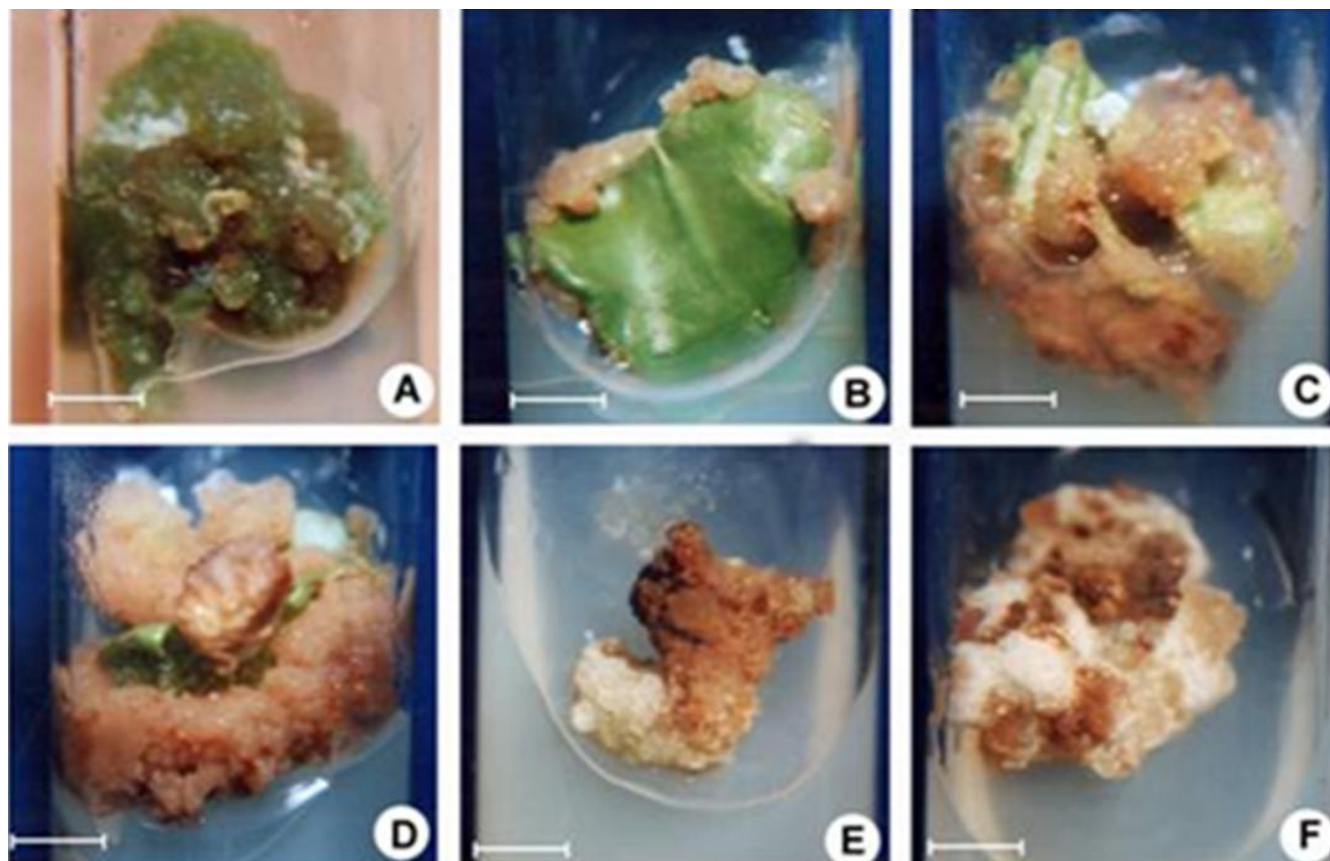
After 6 weeks '+' represents organogenetic ability of callus, '-' non organogenetic ability of callus, NR- No response, '++' indicates moderately organogenetic, '+++' highly organogenetic.

of seedlings, by far the best response was observed using 14-day-old seedling tissues. Subsequent experiments indicated that explants taken from seedlings between 2 and 3 weeks of age were capable of similar high levels of callus and plant regeneration. Use of tissues younger or older than this resulted in lower initial frequencies of callus formation and a marked reduction in the number of explants capable of plant regeneration.

### One stage and two stage method of indirect organogenesis

Highly competent explants such as cotyledonary node, shoot tip induced the formation of callus that also gave rise to adventitious shoots on the same medium without transfer. These explants are not different from those, which gave rise to callus, from which shoots might be obtained in

second medium. One stage process summarizes the growth regulators where caulogenesis occurred on the primary callus (data not shown). Seedling explants like leaves, cotyledon and root (Figure 1) gave rise to adventitious shoots from callus in which two cultural stages have been employed. Stage-1 involved obtaining callus from the explant by culturing on an appropriate combination of media ingredients and growth regulators.



**Figure 1.** Effect of plant growth regulators on callus induction from seedling explants of *S. febrifuga* on MS medium. (A) Dark green morphogenic compact nodular callus from cotyledonary explants on  $5.37 \mu\text{M mg L}^{-1}$  NAA +  $2.22 \mu\text{M}$  BA after 3 weeks (B to D) Brown compact friable calli from cotyledonary explants on  $2.26 \mu\text{M}$  2,4-D (E, F) Brown hard compact cotyledonary calli with  $2.22 \mu\text{M}$  BA +  $4.52 \mu\text{M}$  2,4-D.

Stage-2, callus obtained from original explant is transferred to another medium containing different growth regulators. Adventitious shoots produced were excised and rooted.

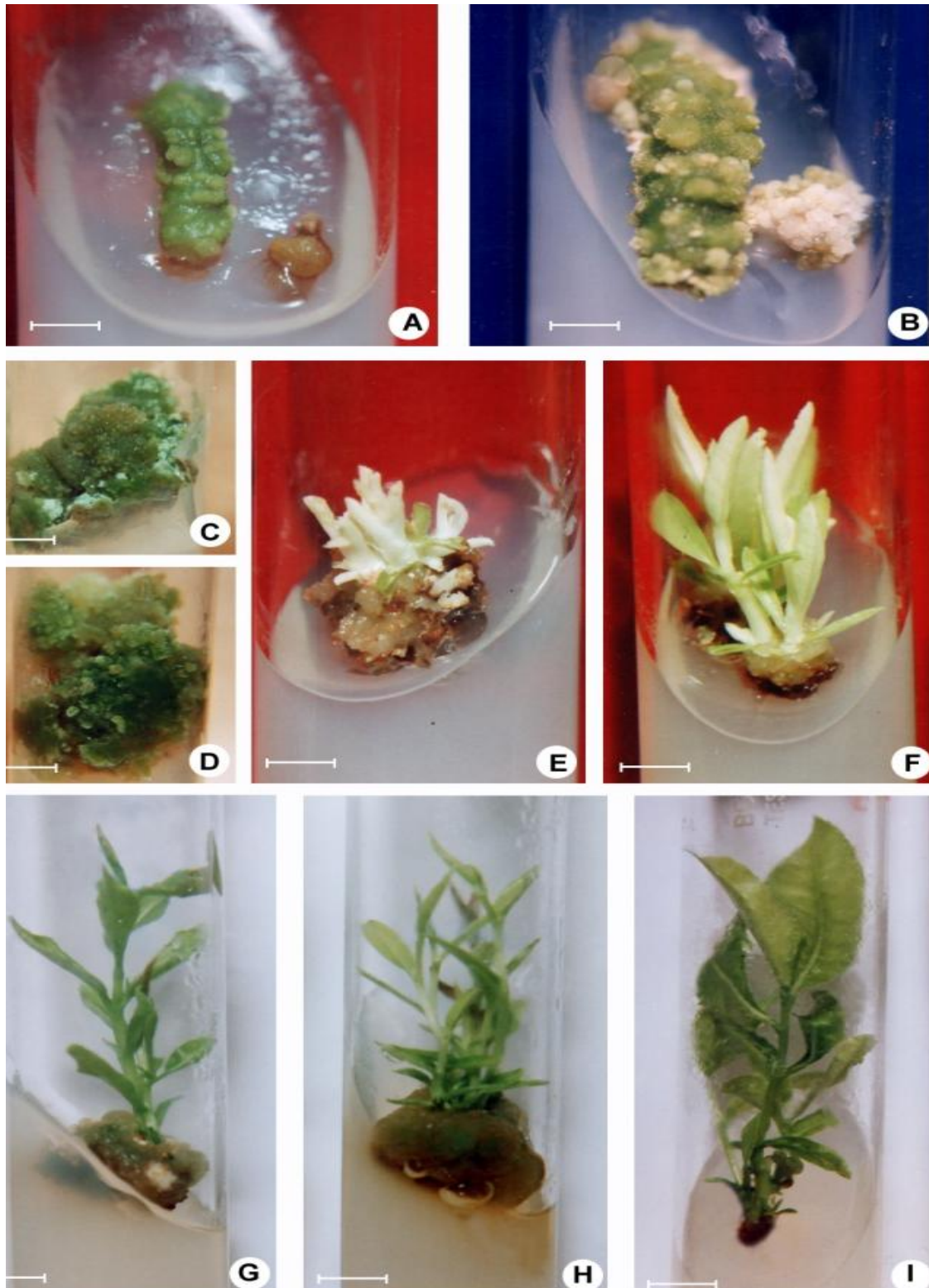
Seedling explants formed callus capable of high frequencies of plant regeneration in media containing both auxin and cytokinin with NAA being the preferred auxin and BA being the preferred cytokinin. The best initial callusing response was observed in MS medium containing a combination of  $5.37 \mu\text{M}$  NAA and  $2.22 \mu\text{M}$  BA for cotyledons (Figure 1A). Callus mostly developed from the edges of the cotyledon and leaves that gradually extend to other parts of the explant (Figure 1B and D). Light/dark green coloured compact nodular calli with high morphogenic capacity was observed from leaf as well as cotyledon on MS medium supplemented with BA + NAA at various concentrations and also on  $15 \mu\text{M}$  of 2-iP. MS medium containing different combinations of 2,4-D/2-iP/BA has not responded better in the production of morphogenic calli from both leaf and cotyledonary explants (Figure 1E and F). NAA in combination with BA was the best phytohormone for the production of morphogenic callus.

Light brown soft friable nodular callus with small green patches was observed from root callus on MS medium fortified with  $0.89 \mu\text{M}$  BA +  $0.2 \mu\text{M}$  NAA. Occurrence of green spots, which are considered meristematic centers, can be a predictor of the capacity of callus to produce shoots (Ishi, 1982; Henni et al., 1997). In all the combinations of BA and NAA tested, light brown soft friable callus was observed with less morphogenic ability. The synergistic action of auxin and cytokinin on callus formation was found to be pronounced *S. febrifuga* and data in Table 1 shows morphogenic callus of explants is induced to grow with relatively low concentration of auxin usually but invariably combined with cytokinin.

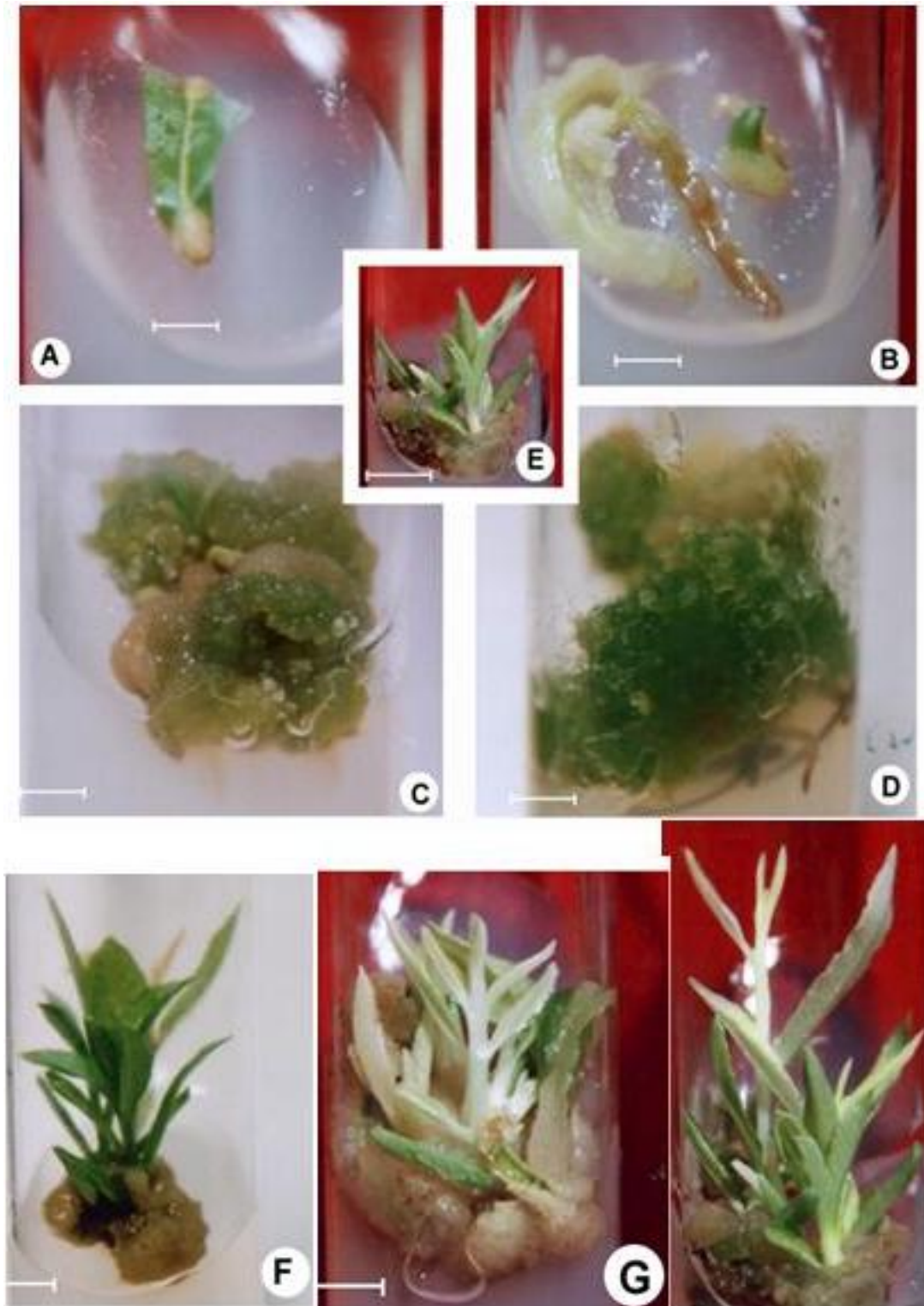
Adventitious shoots are induced to form from the callus cultures at Stage-2 by transferring to a medium containing the weaker auxin (Figures 2 and 3).

#### **Effect of plant growth regulators on indirect shoot morphogenesis from cotyledon and leaf explants**

After subculturing the callus on the mentioned regenerating medium, small amount of green and compact callus



**Figure 2.** Shoot regeneration from cotyledonary callus of *S. febrifuga* on MS medium. (A, B) Green, nodular cotyledonary callus on 4.4  $\mu\text{M}$  BA + 2.69  $\mu\text{M}$  NAA after 2 & 4 weeks (Bar = 2.8, 2.9 mm) (C, D) Green compact and nodular callus developed in subculture on 4.4  $\mu\text{M}$  BA + 2.69  $\mu\text{M}$  NAA after 2 & 3 weeks (Bar = 2.0, 2.8 mm) (E) Shoot bud induction and regeneration on medium fortified with 13.3  $\mu\text{M}$  BA + 0.54  $\mu\text{M}$  NAA after 4 weeks (Bar = 5.2 mm) (F, G) Multiple shoot proliferation and elongation on the same above medium after 6 & 8 weeks (Bar = 5.2, 5.2 mm) (H) Regenerated and elongated shoot kept for rooting after 8 weeks (Bar = 5.0 mm).



**Figure 3.** Shoot regeneration from leaf calli of *S. febrifuga* on MS medium.

(A, B) Creamish compact and loose, friable leaf callus formation on medium supplemented with 2.69  $\mu\text{M}$  NAA after one & three weeks (Bar = 2.5, 2.6 mm) (C, D) Green compact, nodular friable callus developed on subculture on the same above medium after 4 & 6 weeks (Bar = 2.1, 2.9 mm) (E) Shoot bud induction and proliferation from leaf calli on transfer to medium containing 4.4  $\mu\text{M}$  BA + 2.69  $\mu\text{M}$  NAA after 4 weeks (Bar = 6.2 mm) (F) Multiple shoot production on the medium fortified with 4.62  $\mu\text{M}$  KN after 6 weeks (Bar = 3.7, 4.8 mm) (G) Multiple Shoot production, proliferation and elongation from leaf calli on transferring to medium containing 4.4  $\mu\text{M}$  BA + 2.69  $\mu\text{M}$  NAA after 6 weeks (Bar = 6.2 mm).

**Table 2.** Effect of various combinations of plant growth regulators in MS medium on indirect shoot regeneration from cotyledon and leaf calli of *S. febrifuga*.

Plant growth regulators ( $\mu\text{M}$ )				Cotyledon			Leaves		
BA	KN	IAA	NAA	Frequency of shoot regeneration	Mean no of shoots/ explant	Mean length of the shoot (cm)	Frequency of shoot regeneration	Mean no of shoots/ explant	Mean length of the shoot (cm)
8.9	-	-	0.54	64.9 $\pm$ 0.65 <sup>f</sup>	4.4 $\pm$ 0.04 <sup>d</sup>	4.7 $\pm$ 0.11 <sup>d</sup>	50.1 $\pm$ 0.9 <sup>c</sup>	6.8 $\pm$ 0.06 <sup>h</sup>	3.1 $\pm$ 0.10 <sup>ab</sup>
8.9	-	0.57	-	40.3 $\pm$ 0.95 <sup>b</sup>	3.8 $\pm$ 0.12 <sup>bc</sup>	3.4 $\pm$ 0.13 <sup>bc</sup>	63.8 $\pm$ 0.8 <sup>e</sup>	5.9 $\pm$ 0.09 <sup>g</sup>	2.8 $\pm$ 0.05 <sup>a</sup>
13.3	-	-	0.54	75.5 $\pm$ 0.76 <sup>h</sup>	6.6 $\pm$ 0.0 <sup>g</sup>	3.7 $\pm$ 0.11 <sup>c</sup>	69.5 $\pm$ 0.6 <sup>f</sup>	5.2 $\pm$ 0.15 <sup>f</sup>	3.7 $\pm$ 0.09 <sup>de</sup>
13.3	-	0.57	-	56.0 $\pm$ 0.86 <sup>d</sup>	5.0 $\pm$ 0.11 <sup>f</sup>	2.6 $\pm$ 0.13 <sup>a</sup>	74.6 $\pm$ 0.4 <sup>g</sup>	5.0 $\pm$ 0.14 <sup>ef</sup>	3.3 $\pm$ 0.09 <sup>bcd</sup>
-	13.9	-	0.54	36.3 $\pm$ 0.67 <sup>a</sup>	3.6 $\pm$ 0.07 <sup>b</sup>	5.2 $\pm$ 0.08 <sup>ef</sup>	80.1 $\pm$ 0.9 <sup>h</sup>	3.6 $\pm$ 0.12 <sup>c</sup>	4.2 $\pm$ 0.08 <sup>fg</sup>
-	13.9	0.57	-	45.1 $\pm$ 0.79 <sup>c</sup>	4.2 $\pm$ 0.07 <sup>cd</sup>	5.6 $\pm$ 0.08 <sup>f</sup>	84.0 $\pm$ 0.8 <sup>i</sup>	3.0 $\pm$ 0.09 <sup>b</sup>	4.5 $\pm$ 0.09 <sup>g</sup>
-	23.2	-	0.54	40.6 $\pm$ 0.88 <sup>b</sup>	2.8 $\pm$ 0.11 <sup>a</sup>	3.3 $\pm$ 0.11 <sup>bc</sup>	59.6 $\pm$ 0.8 <sup>d</sup>	2.8 $\pm$ 0.11 <sup>b</sup>	3.6 $\pm$ 0.07 <sup>cde</sup>
-	23.2	0.57	-	55.5 $\pm$ 0.76 <sup>d</sup>	3.5 $\pm$ 0.11 <sup>b</sup>	2.7 $\pm$ 0.07 <sup>a</sup>	53.0 $\pm$ 0.6 <sup>c</sup>	2.2 $\pm$ 0.11 <sup>a</sup>	3.2 $\pm$ 0.09 <sup>l</sup>
8.9	13.9	-	0.54	60.8 $\pm$ 0.48 <sup>e</sup>	4.0 $\pm$ 0.13 <sup>bcd</sup>	5.0 $\pm$ 0.15 <sup>de</sup>	48.8 $\pm$ 0.8 <sup>d</sup>	4.2 $\pm$ 0.08 <sup>d</sup>	4.5 $\pm$ 0.11 <sup>g</sup>
8.9	13.9	0.57	-	65.6 $\pm$ 0.68 <sup>f</sup>	3.8 $\pm$ 0.09 <sup>bc</sup>	5.2 $\pm$ 0.12 <sup>ef</sup>	35.3 $\pm$ 0.7 <sup>a</sup>	4.6 $\pm$ 0.07 <sup>de</sup>	3.7 $\pm$ 0.09 <sup>de</sup>
17.8	-	-	0.54	70.0 $\pm$ 0.86 <sup>g</sup>	4.9 $\pm$ 0.15 <sup>ef</sup>	3.0 $\pm$ 0.09 <sup>ab</sup>	37.0 $\pm$ 0.6 <sup>a</sup>	3.2 $\pm$ 0.10 <sup>bc</sup>	4.2 $\pm$ 0.08 <sup>fg</sup>
17.8	-	0.57	-	74.3 $\pm$ 0.67 <sup>h</sup>	4.4 $\pm$ 0.05 <sup>de</sup>	3.5 $\pm$ 0.09 <sup>bc</sup>	41.2 $\pm$ 0.8 <sup>b</sup>	2.9 $\pm$ 0.10 <sup>b</sup>	3.8 $\pm$ 0.06 <sup>ef</sup>

Observation: after 8 weeks. Values represented above are the means of 20 replicates. Mean values having the same letter in each column do not differ significantly at  $P \leq 0.05$  (Tukey Test).

proliferated from the previous callus and from this region shoot induction had taken place. Callus induced from different explant sources was used to investigate their morphogenic responses. Callus segments (approximately 200 mg of FW) were subcultured on to MS media fortified with different growth regulators. Callus derived from all these explants did not show morphogenic responses on MS media free of any growth regulators. The callus thus developed on the mentioned optimal medium was used for further studies to screen the effect of various cytokinins at various concentrations in combination with auxins (NAA and IAA). Addition of growth adjuvants coconut milk (CM) and casein hydrolysate (CH) activated the proliferation of callus rather than promoting its morphogenic ability.

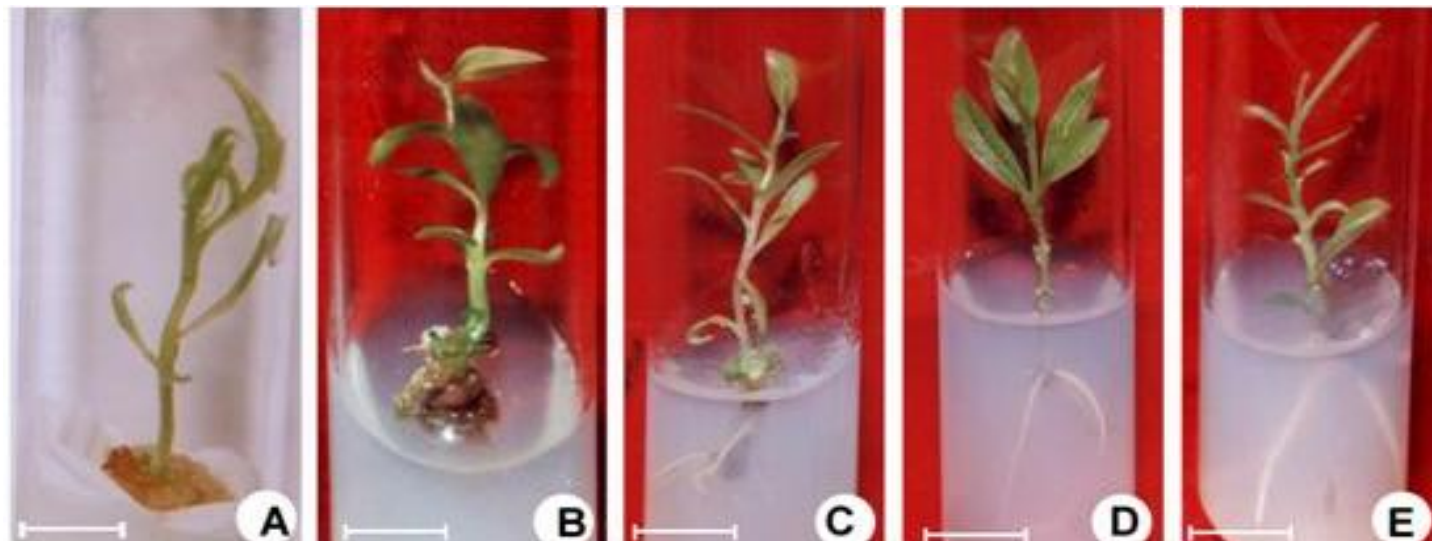
For cotyledonary explants, shoot regeneration occurred 6 to 10 weeks after tissues were transferred from callus induction medium to

regeneration medium. During this period, tissues became gradually more compact, nodular and less translucent with the surface exhibiting green patches, which sometimes covered the entire plant. Adventitious buds then formed in these areas which developed into shoots. Using a two stage regeneration system employing a brief culture period on callus induction medium, it is observed that cotyledon is capable of quite high levels of plantlet regeneration in standard MS medium containing cytokinin (BA) and auxin (NAA) (Table 2). In addition we observed the existence of an optimal age regarding the efficient regeneration from cotyledon, with explants from 2 to 3-week-old seedlings being most suitable.

Various BA and NAA combinations were observed to be effective in promoting regeneration, with best results obtained when explants forming calli (Figure 2A to D) were transferred to regeneration medium containing 4.4  $\mu\text{M}$  BA and

2.69  $\mu\text{M}$  NAA. Among all the concentrations and combinations of various plant growth regulator (PGR) tested, 13.3  $\mu\text{M}$  BA with 0.54  $\mu\text{M}$  NAA proved better in terms of shoot regeneration frequency (75.5%) and production of multiple shoots (6.6) for cotyledonary callus (Figure 2E to H). Callus, which was subcultured onto medium containing high concentrations of BA (17.8  $\mu\text{M}$ ) and Kn (18.5  $\mu\text{M}$ ) failed to show shoot regeneration. However maximum shoot length of 5.6 cm was achieved in MS medium fortified with 13.9  $\mu\text{M}$  Kn + 0.57  $\mu\text{M}$  IAA.

Young leaf explants collected from the seedlings were used for the induction of callus (Figure 3A to D) and subsequent differentiation into shoots on MS medium. The callus induced from abaxial side leaves of *S. febrifuga* in contact with the medium is an excellent source for regeneration of shoots. Many scientists have induced adventitious shoots from *Prunus persica*



**Figure 4.** *In vitro* rooting of *S. febrifuga* on MS half strength medium. (A) Basal callus formation during rooting on medium containing NAA after 3 weeks (Bar = 2.1) (B–E) Rhizogenesis on medium containing 556 mg l<sup>-1</sup> Ca (NO<sub>3</sub>)<sub>2</sub>+ 1.0 mg l<sup>-1</sup> calcium pantothenate +9.8 μM IBA +11.42 μM IAA after 5 weeks (Bar = 5.0, 5.6, 5.8, 5.7 mm).

(Sharma et al., 1999). In the present study, BA (8.9 μM) in combination with NAA (0.54 μM) proved better in the production of multiple shoots (6.8) from leaf callus (Figure 3E to H and Table 2). However IAA proved to be better than NAA when combined with Kn in terms of frequency of regeneration (85.0%) and shoot elongation (4.5 cm). Callus subcultured onto MS medium containing high concentrations of BA (> 13.3 μM) and Kn (> 13.9 μM) failed to show morphogenesis.

#### ***In vitro* rooting of shoots and transfer of plantlets to soil**

Elongated shoots when subjected to rooting displayed shoot tip necrosis as observed during direct organogenesis (Chiruvella et al., 2013) suggesting the micropropagation of this plant is severely hampered by necrosis of shoots causing severe loss (Srivastava and Joshi, 2013; Bairu et al., 2009; Thakur et al., 2008; Martin et al., 2007; Kulkarni and Dsouza, 2000). We circumvented this problem by calcium supply to MS medium in the form of calcium nitrate (556 mg L<sup>-1</sup>) and calcium pantothenate (1.0 mg L<sup>-1</sup>) (Figure 4). Calcium supply not only favored the control of shoot tip necrosis but also induced *in vitro* rooting of shoots (Chiruvella et al., 2011, 2013). Overall, MS medium with IBA fortified with calcium supply was found to be indispensable for rooting of shoots (Figure 4). 70% of regenerated rooted shoots were successfully acclimatized and were morphologically similar to *in vivo* plants.

#### **DISCUSSION**

Seeds are potentially good source to produce disease

free and fungal resistant plants as the disease pathogens cannot penetrate into the seed although the plant is infected. Hence seedling explants were used in current study. Because of *S. febrifuga* medicinal importance and potential to produce valuable secondary metabolites in tissue culture, it is of great interest to develop biotechnological methods to improve the cultivation of *S. febrifuga*. Although the micropropagation of this tree by direct organogenesis has been described by our group (Chiruvella et al., 2013), reports on the indirect organogenesis of this endemic flora have not been explored so far.

Importantly, plant tissue culture is indispensable for improving tolerance studies (such as drought, salt, heat and stress) by inducing somaclonal variation. Hence, it is important to develop an efficient protocol of callus proliferation to start *in vitro* selection for tolerance. Therefore, to obtain a plant regeneration system for a given genotype, it is crucial to screen for their ability to induce callus and regenerate plants. The potential genetic variability associated with this system assumes the importance for genetic improvement and selection strategies, which provides a continuous and constant year round production of plant material. Hence callus mediated plant regeneration is a well recognized technique for the tree improvement by providing somaclonal variants for the selection of new varieties. Shoots regeneration from callus is a re-differentiation process, callus mediated organogenesis *in vitro* is predominant for many plant species (Noel et al., 2002; George, 1993). The interaction of endogenous and exogenously supplied growth regulators is important for shoot regeneration from callus (Torrey, 1961). Cytokinins by themselves can induce shoots.

High cytokinins/auxin ratio is a prerequisite for organogenesis in callus (Srivastava and Rajani, 1999). The decisive factor controlling organogenesis was the



balance of cytokinins and auxins as envisaged (Skoog and Miller, 1957; Torrey, 1961; Nabors et al., 1982). This combination in the present study simultaneously proved a better effect on regeneration of shoots from callus in accordance with previous reports (Tejavathi and Sailaja, 1999; Rey and Mriginski, 1996). This positive effect on growth is explained through enhanced RNA synthesis. In *S. febrifuga* high frequency of shoot regeneration from the callus was achieved with BA along with lower levels of auxins, that is NAA (Figures 2 and 3). The same effect of BA and NAA was reported in *Aeschynomene* (Chen et al., 2003) and *Dioscorea zingiberensis* (Gentile et al., 2002).

The orientation of leaf explant in culture medium can play an important role not only in the response of callus induction and its morphology but also organogenesis from callus (Bhat et al., 1992). Our studies clearly demonstrate that callus derived from leaf showed high frequency of morphogenesis when it was subcultured onto the medium with BA in combination with NAA, whereas BA in combination with IAA induced less frequency of shoot regeneration (Figure 3). Callus regenerated on 2,4-D has not shown any morphogenic sign in subculture as was observed in other systems also (Mc lean, 1992; Jaiswal and Narayan, 1985; Saxena et al., 1997), but is contradictory to the reports (Kumar, 1992; Cerdas et al., 1997). Among all the cytokinins tested, BA induced maximum frequency of shoot regeneration as supported by studies (Bhat et al., 1992; Mclean, 1992; Cerdas et al., 1997).

Altogether, our indirect shoot regeneration system can be used for effective screening and propagation of elite clones, useful for commercial nurseries to produce virus-free plants and agricultural practices that could reduce drought resistance and increase production, germplasm conservation and genetic transformation studies. This serves as an alternative source of regeneration protocol in tissue culture of *S. febrifuga*, as it has high rates of regeneration. Importantly it could be potentially useful for the production of propagules throughout the season constantly. Indirect shoot organogenesis is usually associated with higher genetic variability, resulting in somaclonal variants that might enhance more secondary metabolites. Hence our studies also provide alternative source (callus) to intact plant for extraction of secondary metabolites throughout the year without any seasonal constraints.

## Conclusion

These are the first successful attempts to establish consistent micropropagation using callus from juvenile seedlings of *S. febrifuga*. This technology may provide a valuable tool in a tree improvement program to minimize the pressure on wild populations and contribute to the conservation of valuable flora of the Western Ghats. Our callus culture protocols described will offer the possibility

of producing disease free plants for *ex situ* conservation and secondary metabolism studies.

## ABBREVIATIONS

**BA-6**, Benzyladenine; **CM**, coconut milk; **CH**, casein hydrolysate; **2-iP**, 2-isopentynyl adenine; **IBA**, indole3butyric acid, **IAA**, indole3acetic acid, **KN**, kinetin, **MS**, Murashige and Skoog; **CH**, casein hydrolysate; **PGR**, plant growth regulator.

## Conflicts of interests

This research work has been carried out on the agreement of all the authors, and the paper is submitted after the concurrence of all of them. The authors do not have any conflict of interests.

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