Short Communication

Plant regeneration of *Coscinium fenestratum* (Gaertn.) Colebr. through axenic seed culture and axillary bud culture

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Abstract

In vitro culture methods were studied for *Coscinium fenestratum* (Gaertn) Colebr. (*F. Menispermaceae*), a critically endangered and highly traded medicinal plant, for its conservation and mass propagation. Best surface sterilization of seeds was achieved in axenic seed culture using bavistin (0.3%) for 20 min and streptocyclin (0.1%) for 20 min followed by 0.1 per cent $HgCl_2$, (15 min). Among the different media tested for *in vitro* seed germination, the sterilized sand: coir pith (1:1) soaked with distilled water was found to be better with the highest germination percentage (22.95 %) and lowest germination time (55.68 days). An efficient shoot initiation and multiplication protocol was developed using seedling cotyledonary nodal explant in MS medium supplemented with 0.2mg L^{-1} BA and 0.06 mg L^{-1} 2, 4 - D (4.5 shoots/culture). When nodal segments with an axillary bud from polyhouse grown plants were used as explants, WPM was found to be better than the MS medium for shoot initiation and among the growth regulators, kinetin at 0.4 mg L^{-1} was found to be superior for shoot induction (91.63%). Zygotic embryo excised from GA_3 pre-treated seeds (4000 ppm GA_3 solution for 72 hrs.) when cultured on MS medium in dark condition for 2 weeks followed by exposure to the light condition showed faster development of the embryo, radicle emergence (100 %), plumule emergence (77.78 %) and seedling development (44.44 %).

Key words: Axenic seed culture, Axillary bud culture, Conservation, *Coscinium fenestratum*, Cotyledonary nodal culture, Embryo culture, *Menispermaceae*, Micropropagation.

Coscinium fenestratum (Gaertn.) Colebr. is a medicinally important, perennial woody climber belonging to the family Menispermaceae (Fig.1). It is commonly known as 'tree turmeric' in English and locally as *Maramanjal* in Kerala and Tamil Nadu (Thriveni, 2015). Berberine, a yellow crystalline isoquinoline alkaloid is the main active principle compound present in the plant (Rojsanga et al., 2009). In the traditional Ayurveda and Siddha systems of medicine, the plant has been used mainly for treating diabetes mellitus. In ethno medicine, the stem is used as poultice for cuts and sores, ulcers, fever, jaundice, snakebite, piles, etc. Roots are used as a bitter tonic, for dressing wounds, treating ulcers and dysentery, and also used as stomachic and antiseptic. Pharmacological studies have revealed that *C. fenestratum* extract has antiacne, antiinflammatory, antioxidant, hypotensive, antiplasmodial, antibacterial, antidiabetic, antiproliferative, antihepatotoxic, CNS depressant and analgesic properties (Rai et al., 2013).

Owing to its therapeutical potential, *C. fenestratum* has attained significance as one among the highly traded medicinal plants, which has resulted in the unsustainable overexploitation of the plant from its natural habitats. In addition to this, habitat destruction due to deforestation has led to a

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reduction of the natural population of this species. Moreover, long pre-bearing age, seed dormancy, and viability and regeneration related problems have led to the extinction of this species, and now the plant is listed as a critically endangered species in the IUCN red list of threatened species. *C. fenestratum* is a slow growing woody plant which takes almost 15 years to attain the reproductive stage (Tushar et al., 2008). Seed germination is very low under normal conditions since it exhibits both chemical and mechanical dormancy. In addition to the above factors, dioecious nature of the plant is another issue which demands the presence of male and female plants in close proximity coupled with their synchronized flowering, to get fruiting. These factors in turn have led to the insufficient availability of planting material for conservation and mass propagation of the species.

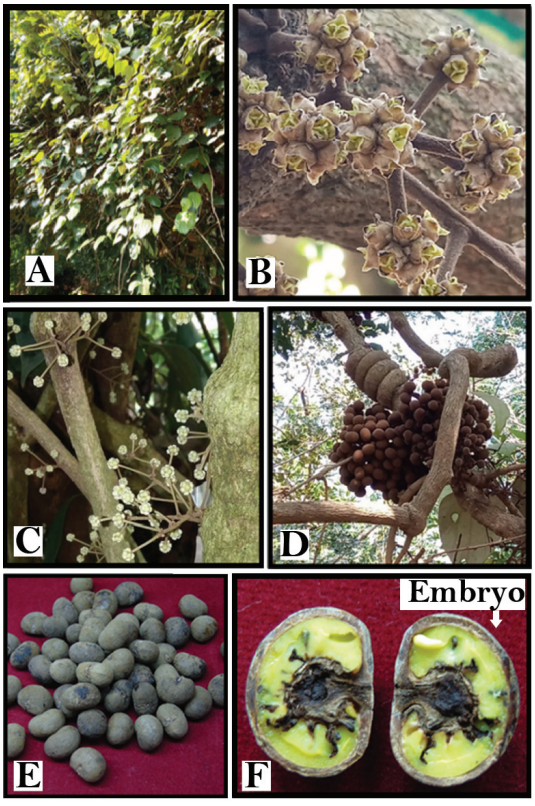

Figure 1. Coscinium fenestratum A) Plant B) Female flower C) Male flower D) Fruits E) Seeds F) Split seed with yellow coloured embryo near the hilum

In conventional methods, the species is chiefly propagated through seeds and vegetative perennial stem cuttings. However, these conventional methods are not adequate to meet the demands of conservation and sustainable utilization of this species. Plant propagation by tissue culture is widely recommended as a biotechnological tool for mass multiplication and conservation of endangered species. Therefore, the development of an *in vitro* protocol for the production of planting material is important to conserve this valuable endangered species and also to assure the germplasm conservation. Hence the present study was undertaken to develop a feasible and reproducible *in vitro* protocol for conservation of *Coscinium fenestratum*(Gaertn.) Colebr.

In axenic seed culture experiment healthy ripened fruits were collected from the mother plant during the ripening season of *C. fenestratum* i.e., July to August. Seeds were extracted from the fruits and adhering pulp was removed.Then a mechanical scarification was given to seeds by rubbing using a sterilized stone to smoothen the hard seed coat. Inside the laboratory, they were air-dried for few hours. Prior to use, seeds were washed with a mild detergent solution for 10 minutes and washed under running tap water for half an hour. Seeds were pretreated with Bavistin 0.3 per cent (w/v) and Streptocycline 0.1 per cent (w/v), each for 20 minutes. Seeds were then soaked in 4000 mg L^{-1} GA_3 solution for 72 hours. These seeds were surface sterilized by different treatments (Table 1) and inoculated in the basal MS media. After exposure to the best surface sterilization method, seeds were cultured on three different media (Table 2). All seeds were incubated in the culture room at 25±2°C temperature in dark condition for germination.

In shoot initiation and multiplication medium standardization experiment cotyledonary nodal explant (from one month old seedling) and nodal segments with axillary bud (from mature mother plant) were taken as explants and brought to the laboratory. Explants were washed with liquid

Table 1. Effect of surface sterilization treatments in the culture establishment of *Coscinium fenestratum* seeds

Treatment No.	Sterilization methods	Culture establishment (%)
T_1	20% (v/v) Clorox solution for 5 min followed by 70% (v/v) ethanol for 2 min	0
T_2	20% (v/v) Clorox solution for 3 min followed by 70% (v/v) ethanol for 1 min	0
T_3	0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 2 min	5.33
T_4	20% (v/v) Clorox solution for 10 min followed by 70% (v/v) ethanol for 5 min	0
T_5	50% (v/v) Clorox solution for 3 min followed by 70% (v/v) ethanol for 1 min	0
T_6	50% (v/v) Clorox solution for 5 min followed by 70% (v/v) ethanol for 2 min	0
T_7	4% (v/v) sodium hypochlorite solution (NaOCl) for 3 min followed by 70%(v/v) ethanol for 1 min	0
T_8	4% (v/v) sodium hypochlorite (NaOCl) solution for 5 min followed by 70% (v/v) ethanol for 2 min	0
T_9	0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 5 min	5.33
T_{10}	0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10 min	10.67
T_{11}	0.1% (w/v) mercuric chloride (HgC_2) solution for 15 min	25.33

detergent for 10 minutes and under running tap water for 30 minutes. Explants were then pretreated with 0.3 per cent (w/v) bavistin and 0.1 per cent (w/v) streptocyclin, each for 20 minutes. Then explants were surface sterilized with 0.1 per cent (w/v) mercuric chloride for 2 minutes and ethanol 70 per cent (v/v) for 1 minute inside the laminar airflow chamber. Cotyledonary nodal explants were cultured on MS media devoid of plant growth hormones or MS media supplemented with either BA or kinetin (0.1 mg L^{-1}, 0.2 mg L^{-1} or 0.4 mg L^{-1}) alone or in combination with 0.06 mg L^{-1} 2,4 - D. Nodal segments with axillary bud as explants were cultured on either MS medium or Woody plant medium devoid of plant growth hormones or supplemented with growth regulators BA or kinetin (0.1 mg L^{-1}, 0.2 mg L^{-1}, 0.4 mg L^{-1}). All the media were supplemented with 1g L^{-1} activated charcoal as an antioxidant and toxic absorbent. The cultures were maintained at 25±2 °C inside the culture room under 16 hour photoperiod.

In embryo culture experiment, mature embryos were excised from the seeds treated with GA_3 (4000 mg L^{-1} GA_3 solution for 72 hours) and non-GA_3 treated seeds and separately cultured. Under the fully aseptic condition, seeds were cut into two halves and bright yellow coloured mature embryos located near the hilum region were excised and cultured on the basal MS medium containing 1g L^{-1} activated charcoal, 3 per cent sucrose, and 0.75 per cent agar without any plant growth regulators. Culture tubes were kept at 25±2°C temperature in dark condition for 2 weeks, and after that developing embryos were exposed to light.

All experiments were laid out in a completely randomized design (CRD). The effect of different treatments was quantified, and the level of significance was determined by the analysis of variance (ANOVA) and the least significant difference (LSD< 0.05) among mean values. Duncan's multiple range test was used to compare the treatment means.

The axenic seed culture study revealed the presence of fungal and bacterial endophytes in the seeds of *C. fenestratum*. In spite of strong surface sterilization treatments, the endophytes living inside the seeds survived and persisted. Hence after inoculation, endophytes emerged and proliferated within the nutrient-rich MS medium(Fig.2). Even though endophytes were not harmful as such, they interfered with the culture establishment by contaminating the media.There are reports of the presence of endophytes in the seeds of plants such as *Pinus monticola* (Ganley and Newcombe, 2006), and in *Lolium multiflorum*, *Festuca arundinacea* and *F. pratensis* (Latch et al., 1987). So far no research results are available on the presence of endophytes in seeds of *C. fenestratum*, whereas Goveas et al. (2011) isolated 41 endophytic fungi from both leaves and stem of *C. fenestratum.*

Sterilization of seeds with $HgCl_2$ significantly reduced the fungal contamination; however bacterial endophytes remained the main contaminant. Clorox and sodium hypochlorite were found to be ineffective to control contaminants. Among the different sterilants tested for surface sterilization of seeds, 0.1% $HgCl_2$ (15 min) was found to be the best agent for the culture establishment with minimum contamination (Table 1). Similarly Warakagoda and Subasinghe (2014) reported 0.1 per cent $HgCl_2$ as the best surface sterilant for *C. fenestratm* seeds.

Among the different media tried for *in vitro* seed germination sterilized sand: coir pith (1:1) media soaked with distilled water was found to be the best and showed the highest germination percentage (22.95%) and lowest germination time (55.68 days) (Fig.3). The other two treatments were found to be not suitable for seed germination, because of high contamination percentage during culture establishment. Sterilized sand: coir pith (1:1) media soaked with liquid MS medium showed a low germination rate (13.91%). It took an average of 79.8 days for the initiation of germination (Table 2). MS media with 1g L^{-1} activated charcoal was found not to be a favourable media for seed germination, because of high contamination percentage due to fungal and bacterial endophytes

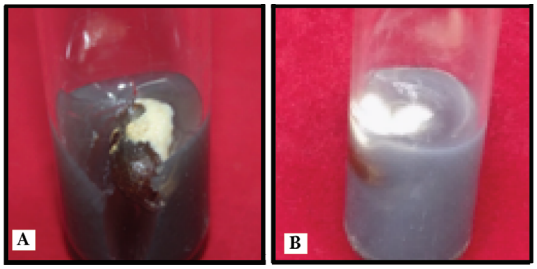

Figure 2. Endophyte contamination in *C. fenestratum* seeds
A) Bacterial endophytes B) Fungal endophytes

Figure 3. Seed germinated in the sterilized sand: coir pith (1:1) soaked with distilled water

which led to full culture loss within a period of one month. This report was confirmatory with that of Warakagoda and Subasinghe (2014). Properly sterilized coir pith soil media soaked with distilled water held the moisture for a long time and acted as a good substrate for seed germination and helped to replace the costly MS media, thereby reducing the media cost and expenses in tissue culture.

An efficient shoot initiation and multiplication protocol was developed using seedling cotyledonary nodal explant. MS media supplemented with 0.06 mg L^{-1} 2, 4-D along with different concentrations of cytokinin, i.e., 0.2 mg L^{-1}BA, 0.2 mg L^{-1} kinetin or 0.4 mg L^{-1} kinetin, promoted shoot initiation (Fig.4), while all other treatments failed to give shoot initiation. A significantly high number of shoots was produced in MS media supplemented with 0.2 mg L^{-1} BA and 0.06 mg L^{-1} 2, 4 - D (4.5 shoots/culture), followed by MS media supplemented with 0.2 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D (3.83 shoots/culture) and MS media supplemented with 0.4 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D (2 shoots/culture). On an average bud initiation was observed within 14 days (Table 3). MS media supplemented with 0.2 mg L^{-1} BA and 0.06 mg L^{-1} 2, 4 - D were stouter and bigger than that obtained from kinetin supplemented media and also produced larger and broader leaves. Among the

Table 2. Effect of different media on the percentage of *in vitro* seed germination and time taken for germination of seeds

No.	Media composition	*In vitro* seed germination (%)	Time taken for *in vitro* seed germination
T_1	MS media with 1g L^{-1} activated charcoal	-	-
T_2	Sterilized sand : coir pith (1:1) soaked with liquid MS media	13.91	79.8
T_3	Sterilized sand: coir pith (1:1) soaked with distilled water	22.95	55.68

Table 3. Effect of different media in cotyledonary nodal explant culture

No.	Media composition	Time taken for shoot induction (days)	Number of multiple shoots
T_1	MS+ BA 0.2mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	13.67	4.50
T_2	MS + Kinetin 0.2mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	14.67	2.00
T_3	MS + Kinetin 0.4mg L^{-1} + 2,4 - D 0.06 mg L^{-1}	14.33	3.83

cytokinin supplemented media, 0.2 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4-D supplemented media produced larger leaves than 0.4 mg L^{-1} kinetin and 0.06 mg L^{-1} 2, 4 - D supplemented media, which showed that when kinetin concentration increased there was a reduction in leaf size.

However, cytokinin alone failed to elicit a response in the present study. Results revealed that the addition of a small amount of auxin with cytokinin enhanced the shoot multiplication. Senarath (2010) reported that *C. fenestratum* seedling epicotyl explant gave the best response when MS medium was supplemented with 1.0 µM kinetin and 0.25 µM 2,4-D, and Joshi and Dhar (2003) reported that 1.0 µM kinetin in combination with 0.25 µM NAA gave the best response for *Saussurea obvallata* seedling epicotyl explant culture.

Among the basal media tried for nodal segments with axillary bud as explants for shoot induction, WPM was found to be better than the MS medium. Among the growth regulators, kinetin at 0.4 mg L^{-1} was found to be superior for shoot induction (91.63%) with WPM basal medium (Fig.5). The period of morphogenic response for shoot induction was faster in the WPM (Table 4). The suitability of WPM over MS media on high-frequency shoot initiation was reported by Lakshmanan et al. (1997) in nodal cuttings of *Ixora coccinea*, Lu (2005) in shoot tip culture of *Vitis thunbergii* Sieb. et Zucc., Raghu et al. (2006) in nodal explant culture of *Tinospora cordifolia*, Alatar et al. (2012) in the nodal segment of *Rauvolfia serpentina* and Warakagoda et al. (2017) in *C. fenestratum.*

GA_3 pre-treatment in *C. fenestratum* seeds prior to embryo culture was found to be effective for seedling development.The embryos excised from the seeds without GA_3 treatment failed to give good response, and there was no proper development of radicles and plumules within a month of observation. Whereas, mature embryos excised from GA_3 pre-treated seeds (4000 mg L^{-1} GA_3 solution for 72 hr),when cultured on MS medium in dark condition for 2 weeks followed by exposure to light condition, showed faster development of the embryo, radicle emergence (100 %), plumule emergence (77.78 %)and seedling development (44.44 %) (Fig.6). The yellow coloured small embryo started to enlarge within a week when kept

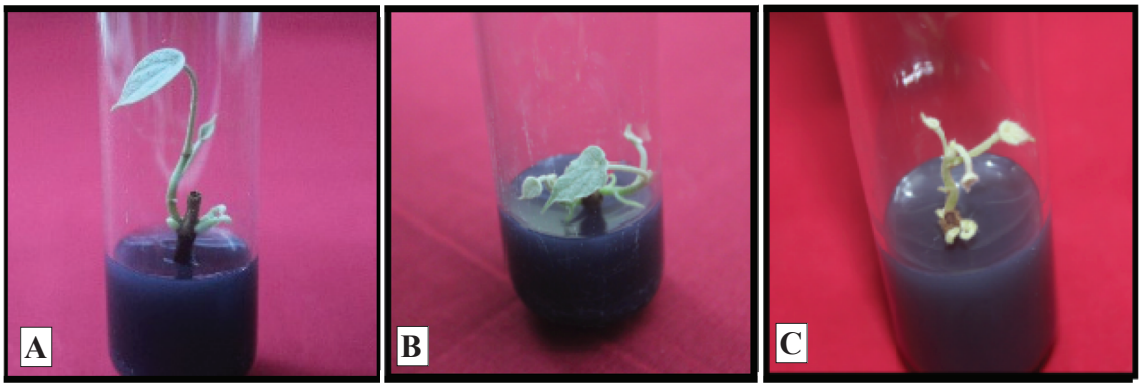

Figure 4. Multiple shoot production in cotyledonary nodal explant After 45 days in MS media supplemented with A) 0.2 BA+ 0.06 2, 4-D, B) 0.2 mg L^{-1} kinetin + 0.06 mg L^{-1} 2, 4 – D, C)0.4 mg L^{-1} kinetin + 0.06 mg L^{-1} 2, 4 – D

Table 4. Effect of different media in nodal segments with axillary bud culture

No.	Media composition	Culture establishment (%)	Shoot induction (%)	Time taken for shoot induction (days)
T_1	MS basal	71.11	*0(1.654)	-
T_2	MS+ BA 0.1mg L^{-1}	73.33	0(1.654)	-
T_3	MS+ BA 0.2mg L^{-1}	77.78	11.36(19.473)	14.33
T_4	MS+ BA 0.4mg L^{-1}	71.11	40.97(39.789)	13.01
T_5	MS + Kinetin 0.1mg L^{-1}	73.33	0 (1.654)	-
T_6	MS + Kinetin 0.2mg L^{-1}	80.00	0 (1.654)	-
T_7	MS + Kinetin 0.4mg L^{-1}	80.00	22.55 (28.190)	12.72
T_8	WPM basal	73.33	0 (1.654)	-
T_9	WPM + BA 0.1mg L^{-1}	80.00	47.67(43.646)	12.72
T_{10}	WPM + BA 0.2mg L^{-1}	77.78	51.26(45.728)	10.70
T_{11}	WPM + BA 0.4mg L^{-1}	82.22	73.29(59.192)	10.10
T_{12}	WPM + Kinetin 0.1mg L^{-1}	77.78	51.28(45.734)	10.47
T_{13}	WPM + Kinetin 0.2mg L^{-1}	77.78	68.44(55.884)	10.81
T_{14}	WPM + Kinetin 0.4mg L^{-1}	80.00	91.63(73.193)	10.57
	CV (%)	9.930	10.939	4.188
	CD (0.05)	NS	5.476	0.851

*Angular transformed values are given in parentheses

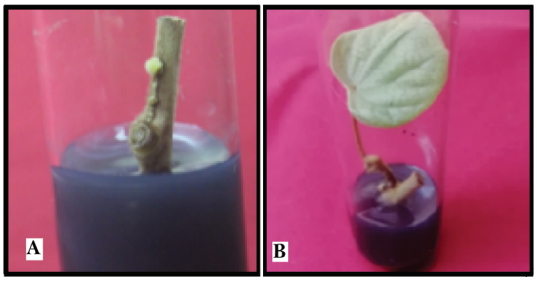

Figure 5. A) Bud produced in the WPM supplemented with kinetin 0.4 mg L^{-1} B) Shoot produced in the WPM supplemented with kinetin 0.4 mg L^{-1}

in dark condition. After two weeks when it was exposed to light the colour changed gradually from yellow to green due to chlorophyll development. Within two weeks, radicle and plumule started to develop and a seedlingwith proper growth was obtained in 60 days. *C. fenestratum* had a very hard seed coat which made excision of embryo difficult under the laminar airflow chamber. The embryo had bipolar nature and damage on any side led to the development of either shoot or root. Compared to seed germinated seedling, seedling developed from embryo culture was found to be smaller in size. These axenic seedlings without microbial contamination could be used as a base material for further micropropagation and conservation of this valuable medicinal plant.

C. fenestratum, which belongs to the critically endangered category of medicinal plants, was found to be a plant of less amenability to tissue culture methods. Even though there is urgent need for conservation of this plant through mass propagation, only less success with tissue culture has been reported so far. A series of trials in the present study resulted in developing a feasible *in vitro* shoot regeneration protocol using cotyledonary nodal

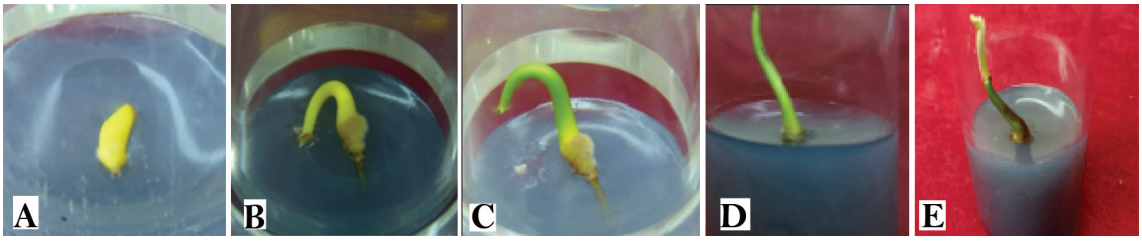

Figure 6. Growth stages of embryo excised from GA_3 pre-treated seed after a) 7 days b) 21 days c) 30 days d) 40 days e) 60 days

explant and axillary bud culture. A mature embryo culture method was also developed.These results could be used as the stepping stone for further development of a high frequency plant regeneration protocol and conservation of this valuable medicinal plant.

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