

Article

Somatic Embryogenesis and Genetic Fidelity Study of the Micropropagated Medicinal Species, *Canna indica*

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Abstract: *Canna indica* Linn. (Cannaceae) is used both as medicine and food. Traditionally, various parts of *C. indica* are exploited to treat blood pressure, dropsy, fever, inflammatory diseases etc. However, to date there is no reliable micropropagation protocol for *C. indica*. We present here a regeneration technique *C. indica* with banana micropropagation medium (BM). BM supplemented with 3% sucrose, 0.7% agar, and 0.17% NH₄NO₃ and different plant growth regulators like BAP (2 mg·L⁻¹) and NAA (0.5 mg·L⁻¹) was found to be effective in inducing callus. BM with BAP (2 mg·L⁻¹) was ideal for somatic embryogenesis and plantlet regeneration. After a period of 3 months, regenerated plantlets were successfully transferred to the field conditions. Appearance of somaclonal variation among the regenerated plants is a common problem which was assessed by DNA fingerprinting. To detect genetic fidelity in *C. indica*, RAPD and ISSR markers were employed. Ten RAPD primers produced 60 amplicons, while 7 ISSR primers generated 45 bands in both *in vitro* plantlets and mother plants. RAPD and ISSR analyses showed no evidence of polymorphism between parent plants and the regenerated plants as all the amplified products were found to be monomorphic.

Keywords: *Canna indica*; callus; somatic embryogenesis; RAPD; ISSR

1. Introduction

Canna, the solitary genus of the family Cannaceae, is a well-known ornamental plant with beautiful flowers. The plant is cultivated extensively throughout the world for its decorative and widely-varying flower colors [1]. In addition, species of *Canna* are also edible [2] and have medicinal properties [3–6]. The common practice for *Canna* propagation is asexual, mainly through multiplication of rhizomes. However, this poses the problem of genetic stagnancy so genetic variation is limited. Varietal improvement of *Canna* lies in genetic manipulation and protoplast fusion [7]. In both the cases a preliminary requirement is a robust micropropagation protocol. Only two groups have attempted this. Sakai and Imai [8] regenerated *Canna edulis* Ker Gawler of callus culture but with limited success. Hosoki and Sasaki [9] produced *C. edulis* with the longitudinal shoot split method. However, no attempts to regenerate has been made to *C. indica* or any other Indian varieties through callus culture have been reported. Therefore, the aim of the present study was to develop and standardize a protocol for regeneration of *C. indica* by regeneration through callus culture.

One major drawback of tissue cultured-grown plants is somaclonal variation (SV) among the micropropagated plantlets. This may reduce the quality of regenerated plantlets considerably, and needs to be checked thoroughly before release. Magnitude of the arrival of SV may depend upon the robustness of the regeneration protocol. One good way of assessing somaclonal uniformity is to study the genetic fidelity. In the present study we employed various fingerprinting techniques like RAPD analysis with random primer-based PCR markers and various ISSR markers to assess genetic fidelity of regenerated plantlets.

2. Experimental Section

2.1. In vitro Germination of Seed

Immature healthy fruit were collected from two-year old *Canna indica* plant grown in a laboratory greenhouse for aseptic culture (accession No. 9590, deposited in the “NBU Herbarium”, Department of Botany, University of North Bengal). The fruit were washed in 1% Tween 20 for 30 min and then rinsed several times with double distilled water (DDW). They were then surface sterilized with 70% alcohol for 45 s, and rinsed several times with sterile DDW under the laminar air flow cabinet. The fruits were finally treated with 0.1% HgCl₂ for 1.5 min and washed several times with sterile DDW to remove traces of HgCl₂. Surface-sterilized fruit were blot dried, and seeds were removed. Single cotyledonous seeds were cut into pieces (usually 2 pieces) on a sterile glass plate and placed on MS medium [10] supplemented with 3% sucrose, 0.42% CaCl₂ and 0.7% agar as a solidifying agent without any growth regulators. Cultures were incubated at 25 °C ± 2 °C with 8 h light exposure. Seeds germinated within 2–3 days. Leaves were excised from 18 ± 2 day old *in vitro* germinated plants and further subcultured in suitable medium for growth and multiplication.

2.2. Callus Induction and Somatic Embryo Production

Leaves of *in vitro* germinated plants were aseptically cultured in banana micropropagation medium (BM) containing various concentrations of growth regulators and supplemented with 1% agar, 30 g·L⁻¹ sucrose and 0.17% NH₄NO₃. The pH of the medium was adjusted to 5.6 ± 0.2 with 1N NaOH or 1N HCl prior to the addition of agar. The media was autoclaved at 121 °C at 15 psi for 20 min.

Callus induction was initiated in media containing different concentrations of 6-benzyl amino purine (BAP) as cytokinin and 1-naphthaleneacetic acid (NAA) or 2,4 dichloro-phenoxy acetic acid (2,4-D) as auxins. Subculturing was done at a regular interval of 2 weeks in the same media having the same hormonal composition [11]. BAP, NAA and 2,4-D at the rates of 1, 2, 3 or 4 mg·L⁻¹ were supplied to observe various stages of *in vitro* callus induction. For somatic embryogenesis, callus tissues were treated with a series of various concentrations of BAP ranging from 1–4 mg·L⁻¹. Controlled cultures with no hormone were also prepared. All of the culture tubes were kept at 25 °C ± 2 °C with a photoperiod of 16 h at 2000–3000 lux light intensity of cool white fluorescent light.

2.3. Histological Observations and Plantlet Regeneration

Somatic embryos were further subjected to histological examination. Histological studies were performed on 15-day-old somatic embryos which were maintained by regular transfer into fresh medium. Transverse and longitudinal sections were dehydrated in graded ethanol (30% to 100%) and stained with safranin and light green [12]. Tissues were suitably mounted in DPX after passing through xylol.

From the embryonic calli, roots and shoots were formed without addition of any other growth regulators by around 16 and 22 days, respectively.

2.4. Hardening and Transfer of Plant to Soil

After 4 weeks of root and shoot *in vitro*, the whole plantlet was carefully taken out of the culture bottle. The roots were washed gently under running tap water to remove the medium completely. The plantlets were acclimatized in the substrates having an autoclaved mixture of soil and sand. Then, the plantlets were transferred to a mixture of sandy soil and manure with at a ratio of 1:1 (v/v) for 30 days for hardening in a greenhouse conditions, and finally to the field. Three sets of 24 plantlets each were planted in a garden soil. The survival percentage of the acclimatized plantlets was recorded.

2.5. Genetic Fidelity Testing of Somaclones

The genomic DNA of *in vitro* regenerated leaf samples of *C. indica* was isolated prior to hardening. This was done by using Genelute Plant Genomic DNA kit (Sigma, St. Louis, MI, USA, Cat# G2N-70). Based on the spectrophotometric analysis, the DNA was diluted to a concentration of 25 ng·μL⁻¹.

A total of 20 RAPD and 10 ISSR primers were screened for PCR amplification (Table 1). Both RAPD and ISSR amplifications were performed using 25 μL of PCR mixture containing 12.5 μL PCR Master Mix 2×, 1.25 μL of primer (0.25 μM) and 2 μL of template DNA (25 ng·μL⁻¹). The PCR reactions were performed on a Perkin-Elmer Thermocycler 2400. The conditions of the thermal cycle for RAPD amplifications were 94 °C for 4 min, followed by 40 cycles of amplification with

1 min denaturation at 94 °C, 1 min annealing at 37 °C, 2 min primer extension at 72 °C, and a final extension at 72 °C for 10 min. The conditions of the thermal cycle for ISSR amplifications were 94 °C for 5 min, followed by 35 cycles of amplification with 45 s denaturation at 94 °C, 1 min annealing at 52 °C, 1 min primer extension at 72 °C, and a final extension at 72 °C for 7 min. The PCR products were separated on 1.5% (w/v) agarose gel with two DNA markers such as λ DNA/*EcoRI*/*HindIII* double digest and a 100 base pair (bp) DNA ladder (GeNei™), and were photographed with Gel Documentation system (UVi).

Table 1. PCR amplification using RAPD (Sl. No. 1–10) and ISSR (Sl. No. 11–17) primers.

Total Bands	Sl. No.	Primer ID	Primer Type	Sequence (5'-3')	Band Size (bp)
7	1	OPA01	RAPD	CAGGCCCTTC	330–1120
6	2	OPA03	RAPD	AGTCAGCCAC	400–1260
6	3	OPA07	RAPD	GAAACGGGTG	500–1550
8	4	OPA11	RAPD	CAATCGCCGT	420–1380
7	5	OPA17	RAPD	GACCGCTTGT	760–1440
5	6	OPB01	RAPD	GTTTCGCTCC	410–1350
6	7	OPF09	RAPD	CCAAGCTTCC	610–1500
7	8	OPG19	RAPD	GTCAGGGCAA	500–1280
4	9	OPH04	RAPD	GGAAGTCGCC	300–1050
4	10	OPN04	RAPD	GACCGACCCA	450–1300
3	11	UBC810	ISSR	(GA)8T	550–800
6	12	UBC815	ISSR	(CT)8G	375–1100
8	13	UBC822	ISSR	(TC)8A	390–1280
6	14	UBC824	ISSR	(TC)8G	450–1110
8	15	UBC825	ISSR	(AC)8T	540–920
5	16	UBC856	ISSR	(AC)8YA	410–980
9	17	UBC873	ISSR	(GACA)4	390–1240

2.6. Statistical Analysis

All the experiments were repeated thrice, and the values were presented as mean \pm standard error. Least significant difference (LSD) values of the treatments were determined between subcultures [13].

3. Results and Discussion

3.1. Establishment of Aseptic Culture

To manage the fungal and bacterial contamination problems a combination of surface sterilants like 70% ethanol for 45 s followed by 0.1% HgCl₂ for 1.5 min showed the best result. There was no significant contamination problem during subculturing and maintaining of explants. Similar observations were noted by Bhattacharya and Sen in the micropropagation of ginger [14].

3.2. Callus Induction

Callus-like structures of the *in vitro* maintained leaves were observed after 15–20 days of culture (Figure 1). Out of various basal media, banana micropropagation media (BM) was found to be the most suitable. BM supplemented with 2 mg·L⁻¹ BAP and 0.5 mg·L⁻¹ NAA assisted profuse callusing.

In contrast, 2,4-D at various concentrations in basal media without any hormone, yielded no result. Therefore, BM with BAP and NAA were the best for callus induction. Fully-developed callus was noticed after 10–15 days of callus induction (Figure 1).

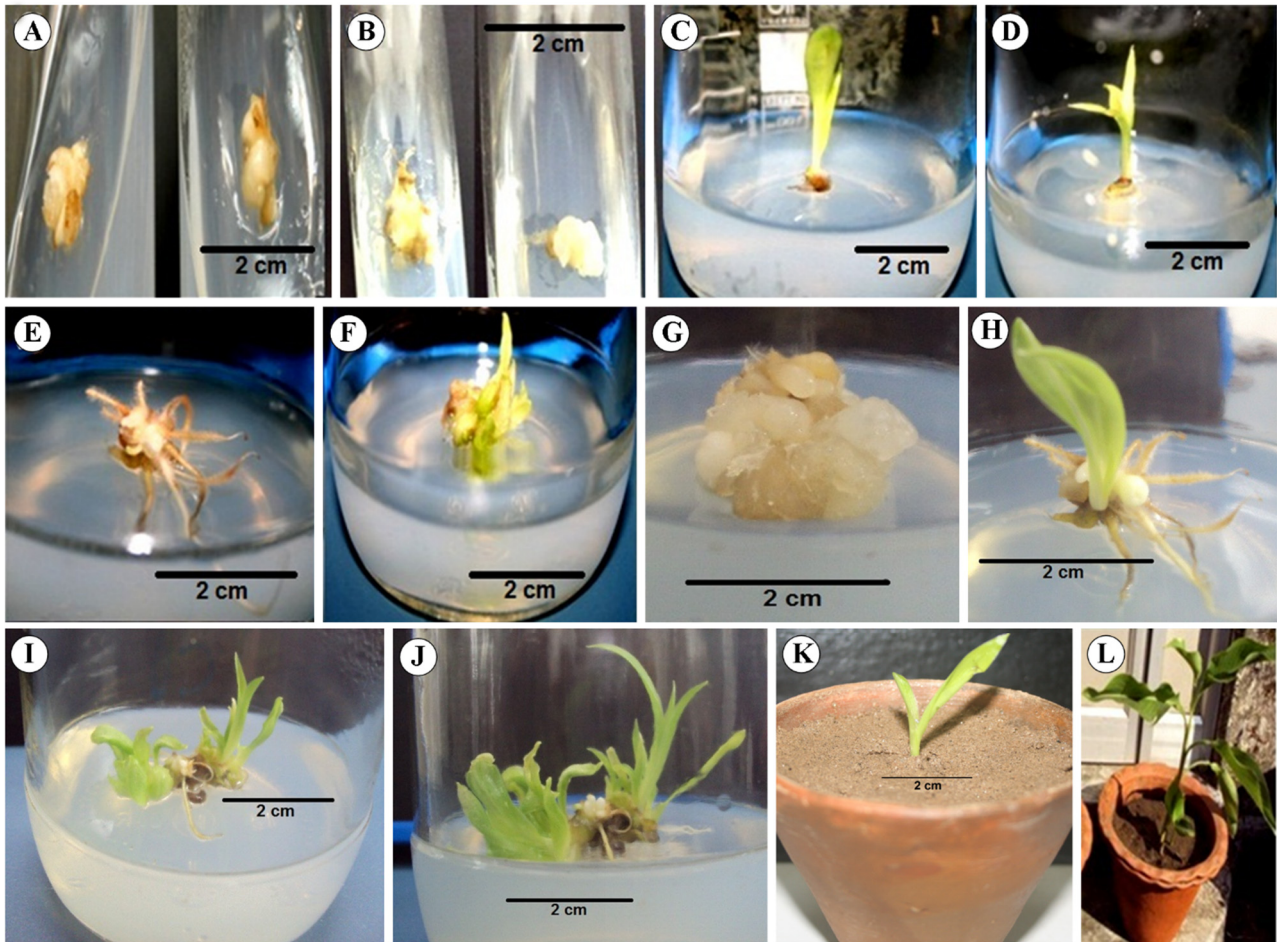


Figure 1. Stages of Callus induction and regeneration: (A) Callus induction; (B) Matured callus; (C) Formation of *in vitro* shoot; (D) Multiple shooting; (E) Profuse *in vitro* rooting; (F) Plantlet regeneration from callus. Stages of somatic embryogenesis and plantlet formation: (G) Somatic embryo formation; (H) *In vitro* shooting and rooting from embryo; (I) Fifteen days old plantlet; (J) Plantlet after 1 month; (K) Acclimatization of plant in clay pot containing mixture of soil and sand; (L) Transfer and hardening of *in vitro* grown plant.

3.3. Somatic Embryogenesis and Regeneration

3.3.1. Somatic Embryo Formation

Similar to the previous experiments, various hormone combinations for somatic embryogenesis were tried and $2 \text{ mg} \cdot \text{L}^{-1}$ of BAP in BM was found most suitable. In fact, auxin (NAA) hindered somatic embryo formation. Somatic embryos were formed after 10–15 days following transfer of callus into BAP-assisted medium (Figure 1). An average 4–5 somatic embryos were produced per

culture vessel. The number of embryos increased (9 ± 3) up to the 3rd subculture, while by the 4th subculture embryo number was reduced (6 ± 2).

3.3.2. Plantlet Regeneration

It was noted that root and shoot from the somatic embryos took place without changing the medium or hormones. In the BAP-assisted BM, rooting was initiated at 19 ± 3 days of somatic embryo formation while shoot development started a little later 28 ± 5 days. Early rooting had no effect on the formation of *in vitro* shoots [15]. However, root and shoot induction varied with different concentrations of BAP. Profuse rooting was observed in all cases. The earliest root and shoot formation (16 and 22 days, respectively) took place at $2 \text{ mg}\cdot\text{L}^{-1}$ of BAP. Perhaps profuse rooting in *Canna* is a general phenomenon for the members of the order Zingiberales as Kambaska and Santilata [16] and Tyagi *et al.* [17] also reported a similar phenomenon in ginger and turmeric, respectively.

3.3.3. Subculturing and Hormone Effect on Shoot Growth

The number and length of shoots varied with the concentration of the phytohormone used (Table 2). The highest number of shoots was observed at $2 \text{ mg}\cdot\text{L}^{-1}$ BAP. The number of shoots increased with BAP up to $2 \text{ mg}\cdot\text{L}^{-1}$, and then gradually decreased thereafter. A higher concentration of BAP may have some inhibitory effect on the formation of shoots [18]. Shoot length also showed a similar pattern; the tallest shoot was recorded at $2 \text{ mg}\cdot\text{L}^{-1}$ BAP. Subsequent subculturing of plantlets also had some effects on the growth rate of shoots (Table 2). The results indicated that *in vitro* growth of *Canna* gradually increased up to a certain limit in response to fresh medium. Decline in regeneration may be the effect of gradual aging and a decrease in totipotency of the explant [19].

Table 2. Effect of subculture and concentration of phytohormones on shoot growth.

Media	BAP ($\text{mg}\cdot\text{L}^{-1}$)	Shoot Length (cm)				
		15 D #	30 D	45 D	60 D	75 D
BM	Control	0.20 ± 0.32	0.48 ± 0.21	1.23 ± 0.58	1.62 ± 0.82	1.81 ± 0.76
	1	0.66 ± 0.38 ^{ns}	1.80 ± 1.22 ^{ns}	3.50 ± 1.61 ^{**}	5.10 ± 0.91 ^{**}	5.90 ± 1.02 ^{**}
	2	1.04 ± 1.07 ^{ns}	3.22 ± 1.81 ^{**}	4.96 ± 0.92 ^{**}	6.50 ± 1.12 ^{**}	7.35 ± 0.84 ^{**}
	3	0.51 ± 0.77 ^{ns}	2.40 ± 1.54 ^{**}	3.20 ± 1.43 ^{**}	4.32 ± 0.98 ^{**}	4.88 ± 0.67 ^{**}
	4	0.42 ± 0.26 ^{ns}	1.70 ± 0.93 *	2.61 ± 0.78 *	3.80 ± 1.31 ^{**}	4.64 ± 1.95 ^{**}
		Shoot No.				
	Control	1.24 ± 0.88	2.31 ± 0.75	3.47 ± 0.92	4.84 ± 0.89	5.16 ± 0.80
	1	3.29 ± 1.12 ^{**}	8.96 ± 0.89 ^{**}	13.44 ± 1.15 ^{**}	18.88 ± 1.26 ^{**}	19.56 ± 0.49 ^{**}
	2	4.38 ± 1.79 ^{**}	11.98 ± 1.93 ^{**}	16.56 ± 0.95 ^{**}	22.24 ± 1.84 ^{**}	24.32 ± 1.77 ^{**}
	3	3.43 ± 1.56 ^{**}	9.01 ± 0.99 ^{**}	12.88 ± 1.77 ^{**}	17.02 ± 0.97 ^{**}	19.42 ± 0.88 ^{**}
	4	2.27 ± 0.44 ^{ns}	7.99 ± 0.77 ^{**}	10.42 ± 1.31 ^{**}	13.62 ± 0.55 ^{**}	15.31 ± 0.81 ^{**}

Standard deviation was calculated on 3 replications, each with sets of 10. #D= No. of days, ^{ns} = not significant, * = significant at 5% level of probability, ** = significant at 1% level of probability.

3.3.4. Acclimatization of *in vitro* Regenerated Plants

The *in vitro* propagated plantlets with a well-developed shoot and root system were successfully transferred to clay pots containing an autoclaved mixture of soil and sand. They were maintained for about 2 months under plastic covers in order to avoid their desiccation (Figure 1). The survival rate of *in vitro* generated plantlets was as high as 80%.

The hardened plantlets were transferred to an outdoor garden. The survival rate of regenerated plantlets was as high as 72% in Canna. However, about 23% of the plantlets turned yellow. After about 2 months following transfer to the garden soil, an average of 5–8 leaves were evident and the height of the plants was around 30 cm.

3.4. Histological Studies of Cultured Embryo

Transverse and longitudinal sections of somatic embryos generated from the leaf tissue revealed the followings under a light microscope in 10× magnification (Figure 2).

- Anthocyanin pigmentation was visualized on the edges of the section in the form of red spots as stained by safranin.
- Primordia of embryo development were observed from the surface of the cotyledon slices, in the form of compact masses of cells arranged sequentially on the edge of section.
- Differentiation and separation of proembryonic globules which were had a well-differentiated epidermis.
- Details of epidermal cell divisions, like periclinal and anticlinal walls and mitotically-active cells were observed.
- Embryonic axes with shoot meristems, cortical tissues, central cylinders and procambial strands were clearly visible.

3.5. Genetic Fidelity among *in vitro* Raised Plantlets

Out of 20 RAPD and 10 ISSR primers, 10 and 7 primers produced amplification, respectively (Table 1). A total of 60 scorable bands with an average of 6 ± 2 bands per primer were obtained from the RAPD analysis, whereas a total 45 bands were produced from 6 primers of ISSR markers showing an average of 7 ± 2 bands per primer. Almost all of the bands generated through both RAPD and ISSR analysis were common in parental genotypes and the regenerated plants of different subcultures. Amplified products exhibited monomorphic patterns across all the subcultures for both markers. Representative RAPD and ISSR profiles are given in Figure 3A and 3B respectively. Though the plantlets were produced indirectly from the leaf tissue through the formation of callus, RAPD and ISSR analyses showed no detectable polymorphisms between parent plants and the regenerated plants. However, clonal fidelity was reported in other Zingiberales like banana and turmeric [17,20,21].

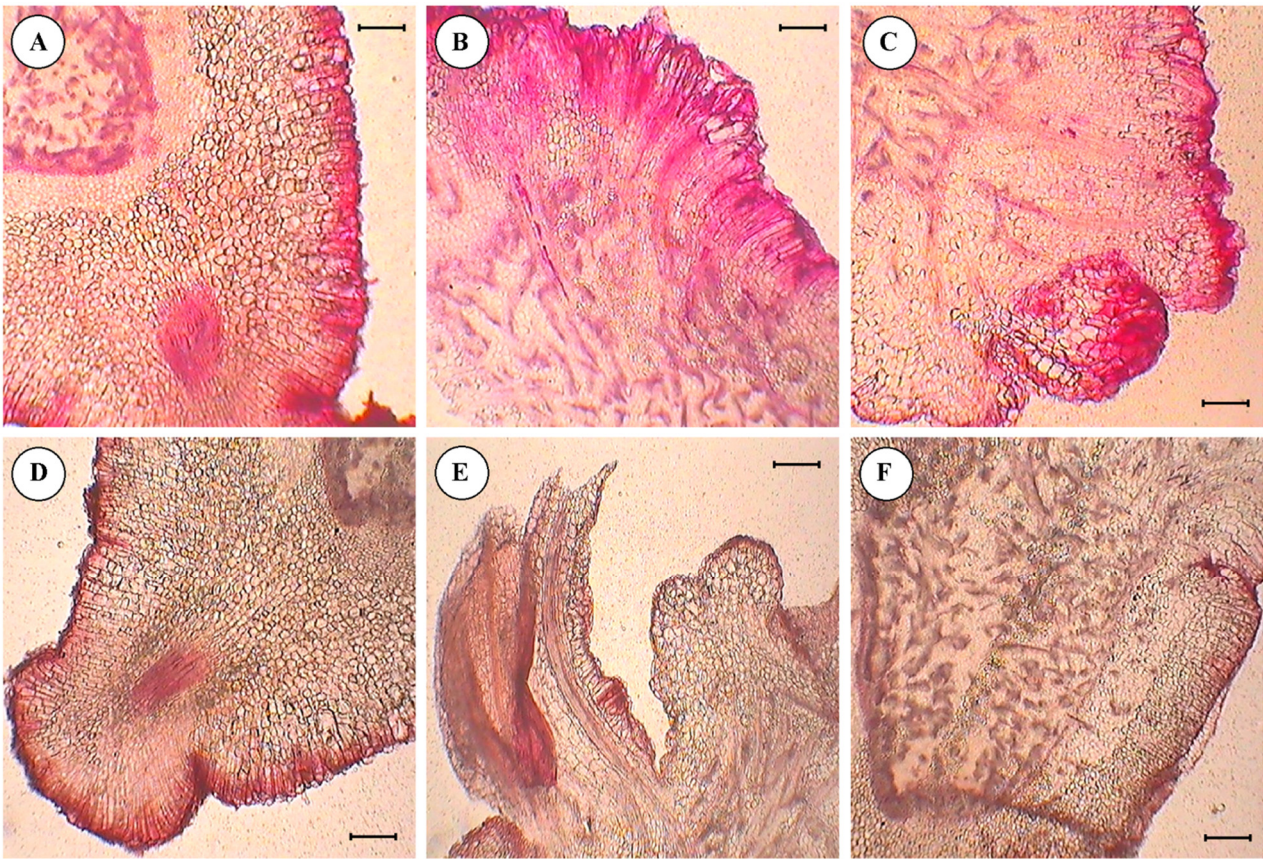


Figure 2. Histology of various stages of embryogenesis observed under light microscope (A) Embryonic axis; (B) & (C) Region of tissue differentiation; (D) Region of embryo induction; (E) Region of shoot primordia; (F) Part of embryonic axis. Scale = 20 μm.

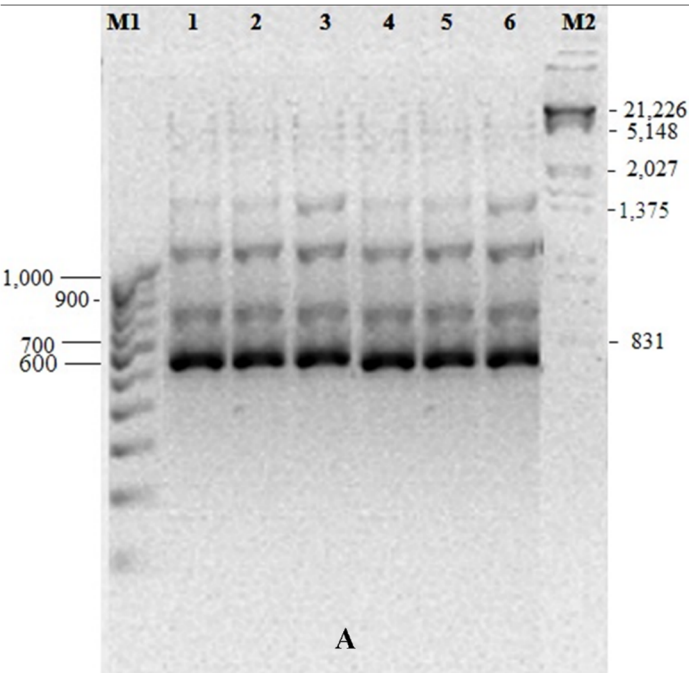


Figure 3. Cont.

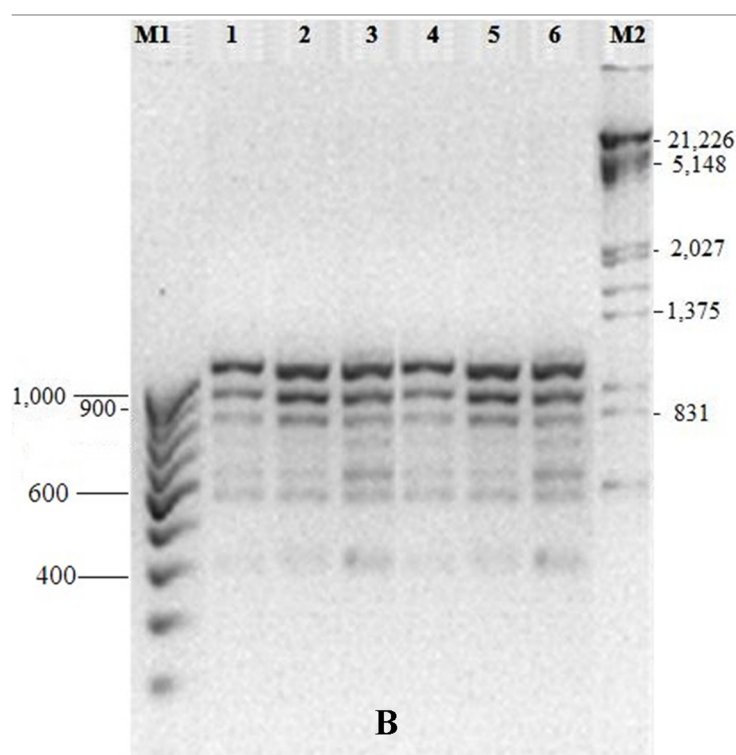


Figure 3. DNA fingerprinting pattern of *in vitro* callus regenerated plantlets of *C. indica*: (A) using RAPD primer OPB01; (B) using ISSR primer UBC815. Lane 2–6: micropropagated plantlets compared with mother plant (lane1); Lane M1: 100 bp molecular marker; Lane M2: λ DNA/*EcoRI*/*HindIII* double digest DNA ladder.

4. Conclusions

The present study is the first report of micropropagation of *C. indica*. It took about 12 weeks to obtain plantlets via somatic embryogenesis. The regenerated plantlets were healthy and RAPD and ISSR analysis showed that they were genetically stable and identical to their parental counterpart. The protocol developed for the rapid regeneration and multiplication of *C. indica* through *in vitro* callus and somatic embryogenesis may be used for the mass propagation of medicinally-important ornamental species.

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Author Contributions

Arnab Sen designed the experiment. Tanmayee Mishra and Arvind Kumar Goyal performed the experiment and wrote the manuscript. All the authors read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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