

Ex Situ Conservation of *Phyllanthus fraternus* Webster and Evaluation of Genetic Fidelity in Regenerates Using DNA-Based Molecular Marker

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Abstract Germplasm storage of *Phyllanthus fraternus* by using synseed technology has been optimized. Synseeds were prepared from nodal segments taken from in vitro-grown plantlets. An encapsulation matrix of 3 % sodium alginate and 100 mM calcium chloride with polymerization duration up to 15 min was found most suitable for synseed formation. Maximum plantlet conversion (92.5 ± 2.5 %) was obtained on a growth regulator-free $\frac{1}{2}$ -strength solid Murashige and Skoog (MS) medium. Multiple shoot proliferation was optimum on a $\frac{1}{2}$ MS medium containing 0.5 mg/l 6-benzylaminopurine (BAP). Shoots were subjected to rooting on MS media containing 1 mg/l α -naphthaleneacetic acid (NAA) and acclimatized successfully. Encapsulated nodal segments can be stored for up to 90 days with a survival frequency of 47.33 %. The clonal fidelity of synseed-derived plantlets was also assessed and compared with that of the mother plant using rapid amplified polymorphic DNA and inter-simple sequence repeat analysis. No changes in molecular profiles were observed among the

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synseed-derived plantlets and mother plant, which confirms the genetic stability of regenerates. This synseed production protocol could be useful for in vitro multiplication, short-term storage, and exchange of germplasm of this important antiviral and hepatoprotective plant.

Keywords Encapsulation · Plantlet conversion · Synseed · *Phyllanthus fraternus* · Genetic stability · Germplasm storage

Abbreviations

BAP	6-Benzylaminopurine
NAA	α -Naphthaleneacetic acid
MS	Murashige and Skoog
RAPD	Random amplified polymorphic DNA
ISSR	Inter-simple sequence repeat analysis

Introduction

Phyllanthus fraternus is an important medicinal herb and belongs to the family Euphorbiaceae. It is widely distributed in tropical and subtropical countries of the world [1]. It is used in traditional and folk medicines for treatment of jaundice, hepatitis, ulcer, and urinary diseases [2]. The antiviral and hepatoprotective properties of *P. fraternus* have increased its demand, which accelerates its indiscriminate removal from natural flora. Moreover, the availability of the plant is subjected to seasonal variation, leading to uncertainty in stable supply throughout the year. There are few reports regarding the tissue culture studies on *P. fraternus*. Shoot regeneration from the node [3–5] and callus developed from hypocotyls [6] have been reported earlier. An efficient multiplication protocol suitable for large-scale multiplication of *P. fraternus* has been previously developed from mature nodal explants in our lab to keep pace with the growing demand for this herb in pharmaceutical industries [7], but no reports are available yet on genetic stability analysis in synthetic seed-derived and hardened *P. fraternus* plants. Ex situ conservation of plant germplasm in the form of field gene banks, seed gene banks, in vitro conservation, and cryogenically preserved tissues is a common practice [8–10]. The artificial seeds also called somatic seeds, synthetic seeds, clonal seeds, synseeds, and somaseeds are defined as an alternative to the botanical seed analogue consisting of somatic embryos surrounded by an encapsulation matrix. However, the currently used broader definition of synthetic seed is an artificially encapsulated somatic embryo, shoot, or any other meristematic tissue which can develop into a plant under in vitro conditions. Synseed technology is an exciting and rapidly growing area of research which deals with in vitro conservation and storage of rare, endangered, and desirable genotypes along with their easy handling and transportation [11, 12]. It is the most effective technique for the conservation of those plant species which produce nonviable seeds and are difficult to propagate by other means [13]. The potential advantages of this technology include a genetically identical, virus-free germplasm, ease in transportation, long-term storage, and low cost of production [14]. Therefore, optimization of appropriate storage conditions and definite storage periods are required to maintain the viability of synseeds for commercial application of this technology. Successful cases of synseed production and plantlet regeneration have been reported for several medicinal plants [15–17]. Singh et al. [18] reported synseed production from shoot tip explants of *Phyllanthus amarus* and found that synseeds can be stored only for 60 days. Moreover, authors have not tested the genetic fidelity of synseed-regenerated plants. The increasing utilization of synseeds for germplasm conservation and propagation necessitates

assessment of genetic stability of conserved propagules following their storage [16]. The limitation of the micropropagation technique is the development of somaclonal variation among regenerated plants, which may affect the phyto-constituent quality and quantity of medicinal plants that hampers the drug development program. Thus, confirmation of the uniformity of genetic content among raw materials utilized in the preparation of medical formulations in the drug development program is prerequisite; otherwise, it will slow down consumers' confidence. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat analysis (ISSR) markers are very commonly used in crop and medicinal plants to analyze genetic stability in tissue culture plants as they are technically simple and quick to perform, requires small amounts of DNA, and have no requirement of prior information about the genome [19, 20]. Some previous workers [21–24] have already used RAPD, SCAR, AFLP, RFLP, and ISSR markers to distinguish *P. fraternus* from its closely related spp. *P. amarus*, *Phyllanthus urinaria*, and *Phyllanthus debilis*. RAPD- and ISSR-mediated DNA polymorphism has been extensively used for detecting polymorphism among in vitro-micropropagated medicinal plants [25, 26]. The objective of the present study is to develop techniques for synseed production, short-term storage, and distribution of *P. fraternus*. This study determines (1) suitable media for plantlet conversion from encapsulated nodal segments; (2) optimal concentration of 6-benzylaminopurine (BAP) for shoot multiplication and plantlet conversion from synthetic seeds under aseptic conditions; (3) optimal concentration of α -naphthalene acetic acid (NAA) for in vitro rooting of shoots developed from synseeds; (4) duration of short-term storage of encapsulated nodal explants at cold (4 °C) and laboratory (24 °C) temperatures, up to which viability can be maintained; and (5) genetic stability analysis among synseed-derived and acclimatized *P. fraternus* plants.

Materials and Methods

Source of Explants

Shoots of *P. fraternus* bearing four to five nodes were cut off from the plants growing in the field, defoliated, and washed under running tap water for 30 min. Further, shoots were soaked in 1 % cetrimide solution with vigorous stirring for 10 min. The shoots were surface sterilized with 0.1 % (w/v) aqueous mercuric chloride solution (HgCl_2) for 10 min and finally rinsed five times with sterilized double-distilled water in laminar flow. Further, surface-sterilized nodal segments were cultured on Murashige and Skoog (MS) basal media. Nodal segments (3–5 mm) from in vitro-differentiated axillary shoots were excised and used as explants for encapsulation.

Media and Culture Conditions

MS media of different concentrations were used during the entire study. The pH of the medium was adjusted to 5.8 using 0.1 N NaOH and 0.1 N HCl prior to adding 0.8 % agar (HiMedia, India). The medium was autoclaved at 121 °C at 1.06 kg cm⁻² for 15 min. All cultures were incubated at 24±2 °C under a 16 h/8 h light/dark cycle, with a light intensity of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from white fluorescent tubes (Philips, India).

Preparation of Encapsulation Matrix and Encapsulation of Nodal Segments

The encapsulation matrix was prepared with varying concentrations of sodium alginate and calcium chloride solution. Sodium alginate was prepared in the range of 1.0, 1.5, 2.0, 2.5, 3,

and 4.0 % (*w/v*) concentration in a liquid MS medium, whereas calcium chloride solution was prepared in the range of 25, 50, 75, 100, and 200 mM concentration in distilled water. Both the gel matrix and complexing agent were autoclaved at 121 °C at 1.06 kg cm⁻² pressure for 15 min. Encapsulation was accomplished by mixing the nodal segments into the sodium alginate solution and dropping these into the calcium chloride solution. The droplets containing the single nodal segment were held for different durations (5, 10, 15, 20, and 25 min) in the calcium chloride solution to achieve polymerization of the sodium alginate. Encapsulated nodal segments were washed five times with sterilized distilled water to remove traces of calcium chloride.

Evaluation of Media for Plantlet Conversion

For conversion into complete plantlets under *in vitro* conditions, encapsulated nodal segments were cultured on liquid and solid MS media of varying strengths. To ensure high-frequency shoot multiplication from encapsulated nodal segments, seeds were cultured on ½ MS medium supplemented with BAP (0.0–2.0 mg/l).

Storage of Synseeds and Assessment of Their Viability After Storage

Encapsulated nodes were kept in a flask capped with a cotton plug, each flask containing 20–25 synseeds. To assess the conversion efficiency of encapsulated nodes after storage, synseeds were stored at cold (4 °C) or culture room temperature (24 °C) for different durations (0, 15, 30, 45, 60, 75, 90 days) without illumination. After storage, synseeds were transferred on a ½ MS solid medium for their germination.

Rooting and Acclimatization

For rooting of microshoots, elongated shoots (3–4 cm) were transferred on MS media containing different concentrations of NAA (0–2 mg/l). Plantlets (3.5–5.0 cm) with well-developed shoots and roots were removed from the culture medium and then transferred to plastic cups containing autoclaved Soilrite, covered with transparent polythene bags, and irrigated daily with 1–2 ml of sterilized MS salt solution for 1 week followed by sterilized tap water; the plants were maintained in the culture room at 24±2 °C and 16-h/day illumination of 20 μmol m⁻² s⁻¹ provided by cool-white fluorescent tubes. After 2 weeks, the polythene bags were gradually removed, and the plants were kept in the culture room for another 2 weeks. The acclimatized plantlets were further transferred to pots containing autoclaved garden soil and sand (1:1) and kept in greenhouse conditions before transfer into the field.

Genetic Stability Analysis

Molecular screening of the micropropagated plants was performed by RAPD and ISSR techniques. Genomic DNA from the mother plant and 16 randomly selected acclimatized plants regenerated from synseeds stored under different storage conditions (4 °C, eight plants; 24 °C, eight plants) were subjected to RAPD and ISSR analyses. Young leaves were used for extracting DNA by cetyltrimethyl ammonium bromide (CTAB) with some modifications in protocol [27]. The quantity and quality of the isolated DNA were determined using a Dyna Quant 200 Fluorometer and 0.8 % agarose gel stained with ethidium bromide. A set of 15 RAPD and 15 ISSR primers supplied by Operon Technologies Inc., CA, USA, were used for initial screening. Amplification was performed using 25 μl PCR mixture consisting of 2.5 μl

10× buffer (GeNei™, Bangalore, India), 1.0 µl dNTP (10 µM: 2.5 µM each of the dNTPs viz. dCTP, dATP, dGTP, and dTTP) (GeNei™), 0.2 µlTaq polymerase (GeNei™), 1.0 µl DNA (approximate 25 ng/µl), 1.0 µl primer (5 pM), and 19.2 µl Milli-Q water. Amplifications were carried out in an iCycler™ Thermal Cycler (Model 4.006, Bio-Rad, Hercules, CA, USA) with an initial denaturation of DNA at 94 °C for 5 min, followed by 1 min denaturation at 94 °C, 1 min annealing at 35 °C, and 2 min extension at 72 °C. The reaction continued for 45 cycles followed by a final extension of 3 min at 72 °C. PCR products were loaded with 5 µl of bromophenol blue and separated on 1.2 % agarose gel (HiMedia, Mumbai, India) in 1.0 % TAE buffer (40 mM Tris acetate, pH 8.2; 1 mM EDTA) using a Minipack-250 electrophoresis system (GeNei™) at 50 V for 3 h. After electrophoresis amplicons were photographed under ultraviolet light using gel documentation system (Syngene Gel Doc, Syngene, Synoptics Ltd., UK). To assess the consistency of band profiles, PCR amplification was carried out three times. The size of the amplicons was estimated by comparison with a 1-kb DNA ladder (GeNei™, Bangalore, India).

Statistical Analysis

Experiments were set up in a completely randomized block design, and each experiment usually had three replicates. Each treatment has 20 beads. The frequency of plantlet conversion was calculated as the percentage of encapsulated nodal segments showing well-developed shoots and roots out of the total number of nodal segments encapsulated. Analysis of variance (ANOVA) was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Duncan's new multiple range test (DMRT) at the $P < 0.05$ level [28].

Results

Preparation of Synseeds

The assessment of the effects of various concentrations of sodium alginate and calcium chloride are prerequisite in order to standardize the preparation of characteristic beads. Encapsulated beads differ considerably in texture, shape, and outline depending on the concentration of the gelling agent (sodium alginate 1–4 %) and complexing agents (calcium

Table 1 Effect of sodium alginate concentration on plantlet conversion (%) from encapsulated nodal segments of *P. fraternus* after 4 weeks of culture

Sodium alginate (% w/v)	Plantlet conversion (%)
1.0	Fragile beads
1.5	63.3±3.3 ^a c
2.0	76.5±1.5 ^a b
2.5	81.65±1.65 b
3.0	92.5±2.5 a
4.0	48.5±1.5 d

Data represent the mean±SE of three independent experiments, each with 20 beads. Mean values followed by the same letters are not significantly different according to Duncan's multiple range test at $P < 0.05$; CaCl₂ solution of 100 mM concentration was used

^a Soft to handle

Table 2 Effect of calcium chloride concentration on plantlet conversion (%) from encapsulated nodal segments of *P. fraternus* after 4 weeks of culture

Calcium chloride (mM)	Plantlet conversion (%)
25	Fragile beads
50	Fragile beads
75	62.25±1.75 ^{a b}
100	92.5±2.5 a
200	41.5±1.5 c

Data represent the mean±SE of three independent experiments, each with 20 beads. Mean values followed by the same letters are not significantly different according to Duncan's multiple range test at $P<0.05$; sodium alginate solution of 3 % was used

^a Soft to handle

chloride 25–200 mM) (Tables 1 and 2). An encapsulation matrix of 3 % sodium alginate and 100 mM calcium chloride was found best for ideal bead formation (Fig. 1a). Sodium alginate concentrations below 3 % were not suitable for encapsulation because the resulting beads were without a defined shape and too soft to handle, while at higher concentrations, the beads were too hard which caused considerable delay in regeneration (Table 1). Similarly, the concentration of calcium chloride also affected the ideal bead formation (Table 2). CaCl_2 solution of 100 mM concentration was ideal for polymerization during the formation of beads. At lower concentrations of CaCl_2 , the beads were fragile, and at higher concentrations, the beads were very compact and hard. The treatment duration of CaCl_2 solution has significant impact on ideal bead formation. The treatment duration of 15 min was best for synseed formation, which resulted in an isodiametric and compact bead. At short treatment durations (5 or 10 min), the beads were too soft and fragile and were difficult to handle, while long treatment durations (20 or 25 min) resulted in hard synseed formation with poor germination.

Plantlet Conversion from Synthetic Seed

Different media (liquid and solid) of various strengths ($\frac{1}{2}$ MS, $\frac{1}{4}$ MS, MS) significantly affect the conversion efficiency of synseeds into plantlets (Table 3). Solid media were better than liquid media for plantlet conversion with well-developed shoots and roots (Fig. 1b–d). Growth regulator-free solid $\frac{1}{2}$ MS medium was best for maximum frequency of conversion (92.5 %) of synthetic seeds, while conversion frequency was lower at full-strength MS medium and lowest at $\frac{1}{4}$ MS medium (Table 3). Thus, it appears that media strength has a significant effect on the frequency of conversion of beads. To evaluate the effect of BAP on conversion and shoot multiplication of encapsulated nodes, synseeds were cultured on $\frac{1}{2}$ MS medium supplemented with different concentrations of BAP (0–2 mg/l) (Fig. 2). Analysis of variance revealed that there is no significant difference in conversion frequency of synseeds and number of shoots per responding explants gradually decreased with increase in concentration of BAP. Maximum frequency of regeneration (99 %) with maximum number of shoots (5.24±0.09) was achieved at 0.5 mg/l BAP-supplemented MS medium. However, there was no rooting in BAP-containing media.

Rooting and Acclimatization

To achieve complete plantlet development, microshoots were transferred on MS media containing different concentrations of NAA (0–2 mg/l). It was observed that there was increase



Fig. 1 Plantlet regeneration from the encapsulated nodal segment of *P. fraternus*. **a** Synthetic seeds formed by encapsulation of nodal segments using 3 % sodium alginate and 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. **b** Germination of encapsulated nodes cultured on the $\frac{1}{2}$ MS liquid medium. **c** Germination of encapsulated node cultured on the $\frac{1}{2}$ MS solid medium. **d** Shoot and root emergence from the encapsulated node on the $\frac{1}{2}$ MS solid medium. **e** An acclimatized plantlet. **f** Hardened plants of *P. fraternus* developed from synthetic seeds transferred into the field

in the rooting of microshoots with increase in the concentration of NAA and after optimum concentration (1 mg/l), rooting frequency was found to decrease (Fig. 3). Rooting frequency (65 %) was maximum at 1 mg/l NAA, with highest average number (4.16 ± 0.44) and length of roots (2.19 ± 0.24). Rooted plantlets were successfully transferred from culture tubes into plastic cups containing autoclaved soilrite, covered with transparent polythene bags, and irrigated daily with 1–2 ml of sterilized MS salt solution for 1 week followed by sterilized tap water; the plants were maintained in the culture room for 2 weeks. The polythene bags were gradually removed, and the plants were kept in the culture room for another 2 weeks. The acclimatized plantlets (Fig. 1e) were further transferred to pots containing autoclaved garden soil and sand (1:1). Acclimatized plantlets were further transferred into the field (Fig. 1f) with 85 % survival. All plantlets survived and flowered normally and were indistinguishable morphologically from the donor plants.

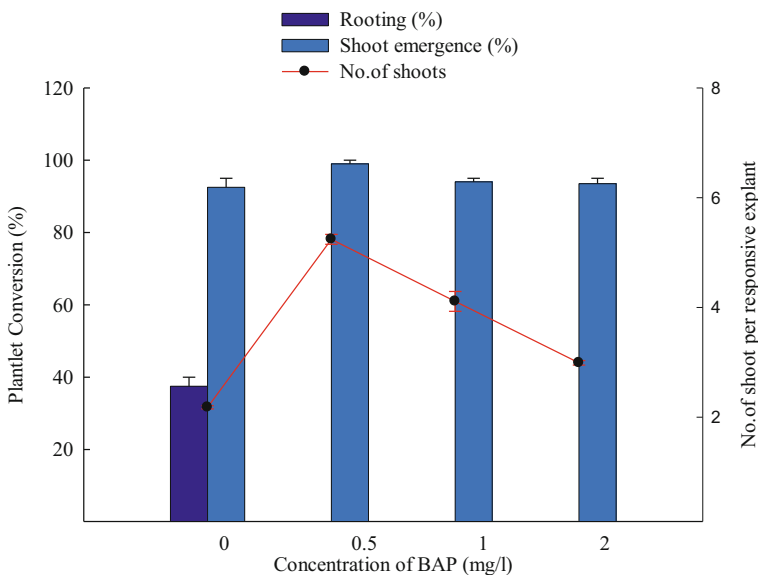
Table 3 Effect of different strengths of liquid and solid MS media on plantlet conversion from encapsulated nodes of *P. fraternus*

Media	Liquid			Solid		
	Plantlet conversion (%)	Average shoot length (cm) (mean±SE)	Average root length (cm) (mean±SE)	Plantlet conversion (%)	Average shoot length (cm) (mean±SE)	Average root length (cm) (mean±SE)
MS	40±2.88 e	1.58±0.12 cd	0.85±0.06 c	68.33±1.75 c	2.7±0.07 b	1.8±2.6 b
½ MS	87.5±2.33 b	2.8±0.15 b	1.48±0.18 b	92.50±2.53 a	3.72±0.10 a	2.6±0.25 a
¼ MS	25.66±2.35 f	1.31±0.10 d	0.94±0.04 c	55±2.88 d	1.78±0.04 c	0.61±0.08 cd

Data represent the mean±SE of three independent experiments, each with 20 beads. Mean values followed by the same letters are not significantly different according to Duncan's multiple range test at $P<0.05$

Storage of Synseeds and Assessment of Their Viability

P. fraternus synseeds were stored for 15, 30, 45, 60, 75, and 90 days at 4 and 24 °C in the dark on an MS medium lacking sucrose. After each storage period at different temperatures, encapsulated nodal segments were further cultured on a ½ MS medium for conversion (Table 4). Analysis of variance of data showed that survival and conversion of encapsulated nodal segments decreased significantly with increased storage temperatures as well as storage duration. The longer duration of storage of encapsulated nodal segments caused significant reduction in plant recovery. Our findings are in close accordance with some earlier works [29, 30, 17]. Synseeds stored at low temperature (4 °C) were superior in terms of their conversion capacity than those stored at culture room temperature (24 °C). The conversion frequency was maintained up to 34 and 47 % after 90 days of storage at 24 and 4 °C, respectively. Encapsulated nodal segment storage at 4 °C was most favorable for the recovery of plantlets.

**Fig. 2** Response of encapsulated nodes cultured on the MS medium supplemented with different concentrations of BAP

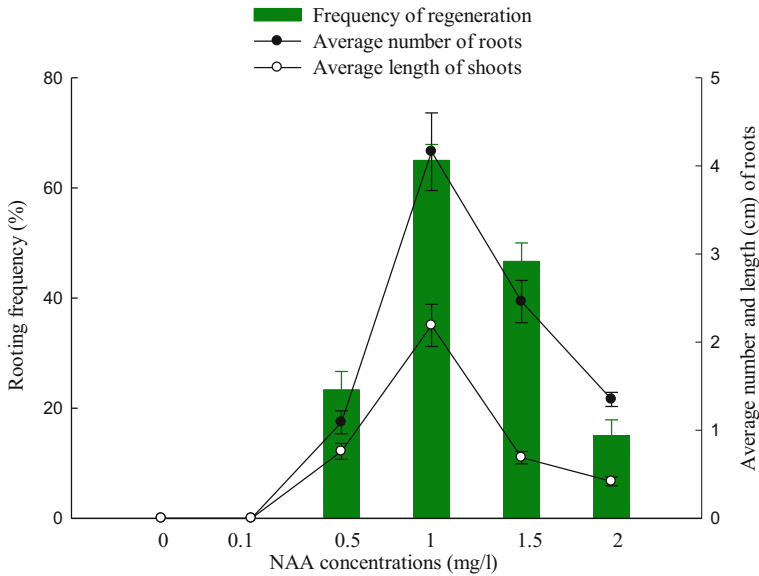


Fig. 3 Rooting of plantlets derived from synthetic seeds of *P. fraternus* cultured on MS medium supplemented with NAA

Genetic Stability Analysis

To confirm the genetic fidelity of regenerated plants from synseeds, 16 randomly selected synseed-derived acclimatized plants of *P. fraternus* stored for 90 days at 4 and 24 °C along with the mother plant were subjected to RAPD and ISSR analyses. Well-resolved, clear, and distinct banding patterns were manually scored from the gel profiles and included for final analysis. Bands with the same mobility were treated as identical fragments, and weak bands were excluded from the final analysis. These plants showed no apparent differences among them and with the mother plant as evident in the RAPD and ISSR analyses. The regenerated plants were similar in morphology to the mother plant. About 15 RAPD primers were tested for amplification, of which 10 produced clear and scorable amplification products during repetitions (Table 5; Fig. 4). The number of scorable bands for each primer varied from three to nine with an average of 5.1 bands per primer. These 10 RAPD primers produced 51 distinct

Table 4 Effect of different storage durations and temperatures on plantlet conversion from encapsulated nodes of *P. fraternus*

Storage duration (days)	Plantlet conversion (%)	
	4 °C	24 °C
0	92.66±1.45 a	92.66±1.45 a
15	86.00±1.0 b	81.66±1.66 b
30	80.66±0.66 c	77.66±1.45 b
45	74.0±2.08 d	67.66±1.5 c
60	62.33±1.45 e	57.66±1.45 d
75	56±1.0 f	50.66±0.66 e
90	47.33±1.45 g	34.33±2.33 f

Data represent the mean±SE of three independent experiments, each with 20 beads. Mean values followed by the same letters are not significantly different according to Duncan’s multiple range test at $P<0.05$

Table 5 RAPD primers amplified in *Phyllanthus fraternus*

Primer	Primer sequences	Total no. of bands amplified	No. of scorable bands per primer	No. of polymorphic bands
OPA-08	GTGACGTAGG	85	5	2
OPA-09	GGGTAACGCC	119	7	0
OPA-10	GTGATCGCAG	68	4	0
OPC-08	TGGACCGGTG	119	7	3
OPL-12	GGGCGGTA CT	51	3	1
OPL-13	ACCGCCTGCT	85	5	0
OPL-14	GTGACAGGCT	153	9	0
OPO-02	CCTCTGACTG	85	5	0
OPO-05	CCCAGTCACT	51	3	0
OPO-06	CCACGGGAAG	51	3	0
Total		867	51	6

and scorable bands in the size range of 250–2,200 bp. A total of 867 bands were generated from the mother plant and 16 in vitro-raised clones, out of which 861 were monomorphic; only

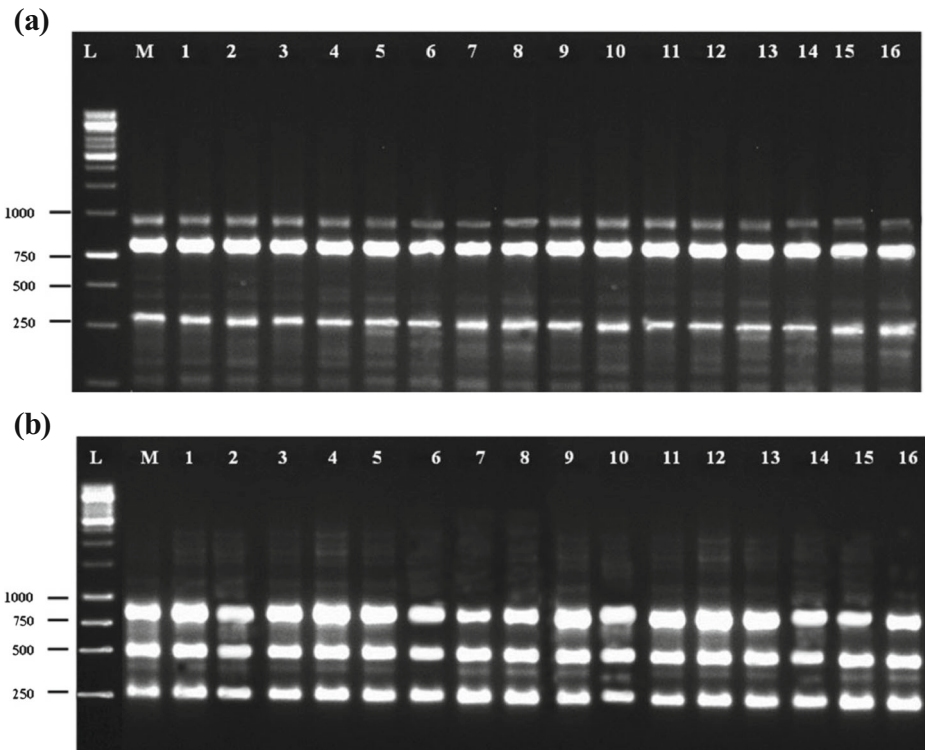


Fig. 4 RAPD amplification pattern obtained with primer a OPO-5 and b OPO-6 for the mother plant (*M*) and the randomly selected plants raised from synthetic seeds (*1–16*) after 90 days of storage under slow growth conditions. *Lane L* molecular marker, *lane M* mother plant, *lane 1–8* the plants grown from synthetic seeds stored at 4 °C, *lane 9–16* the plants grown from synthetic seeds stored at 24 °C

six were polymorphic. Morphologically, all the clones were similar to the mother plant, indicating no variations. Out of 10 RAPD primers, seven primers showed a monomorphic banding pattern within in vitro-raised clones and the mother plant, whereas polymorphic bands were detected with three RAPD primers. No RAPD polymorphism was observed among the mother plant and the plantlets regenerated from synseeds after 90 days of storage under different growth conditions (Fig. 4a, b). For ISSR analysis, 15 ISSR primers were screened, out of which 13 primers gave clear, unambiguous, and reproducible bands (Table 6; Fig. 5). The number of scorable bands for each primer varied from one to three with an average of 1.5 bands per primer. The total number of bands generated from the mother plant and 16 in vitro-raised clones were 255, and there was no polymorphism among them. Such lower frequencies of polymorphic banding patterns have also been revealed by earlier workers in other medicinal plants viz. *Melia azedarach* [31], *Cineraria maritima* [16], and *Rauvolfia serpentina* [32] regenerated from synseeds after storage. Thus, data revealed that plants regenerated from encapsulated microshoots resemble the mother's genetic profiles, based on RAPD and ISSR profiles, after 90 days of storage.

Discussion

A successful propagation system routed through encapsulation is based on the selection of suitable plant parts as the starting plant material, the critical evaluation of factors affecting the gel matrix formation, and the optimization of the process of germination for plant retrieval. Moreover, the optimization of appropriate storage conditions for synseeds, as well as the assessment of fidelity of regenerated plants from synseed after storage, is also required. The concept of synthetic seeds or synseeds was based only on the encapsulation of somatic embryos that could be handled like a real seed for transportation and storage. But, in recent years, the encapsulation of nonembryogenic vegetative propagules has also been employed as a suitable alternative to somatic embryos [33]. The main advantage of using nonembryogenic

Table 6 ISSR primers amplified in *Phyllanthus fraternus*

ISSR primer	Nucleotide sequence (5'–3' motif)	Annealing temperature (°C)	Number of scorable bands per primer	Total no. of bands amplified
UBC-812	GAGAGAGAGAGAGAGAA	50.9	1	17
UBC-815	CTCTCTCTCTCTCTG	55.0	1	17
UBC-822	TCTCTCTCTCTCTCA	55.0	0	0
UBC-834	AGAGAGAGAGAGAGACYT	55.0	0	0
UBC-835	AGAGAGAGAGAGAGGYC	55.0	2	34
UBC-836	AGAGAGAGAGAGAGACYA	55.0	3	51
UBC-840	GAGAGAGAGAGAGAGAY	58.0	0	0
UBC-842	GAGAGAGAGAGAGAGAYG	59.1	1	17
UBC-843	CTCTCTCTCTCTCTRA	53.2	1	17
UBC-844	CTCTCTCTCTCTCTRG	50.9	3	51
UBC-850	GTGTGTGTGTGTGTCTC	60.0	1	17
UBC-853	TCTCTCTCTCTCTCRT	55.0	1	17
UBC-854	TCTCTCTCTCTCTCAGG	58.0	1	17
Total			15	255

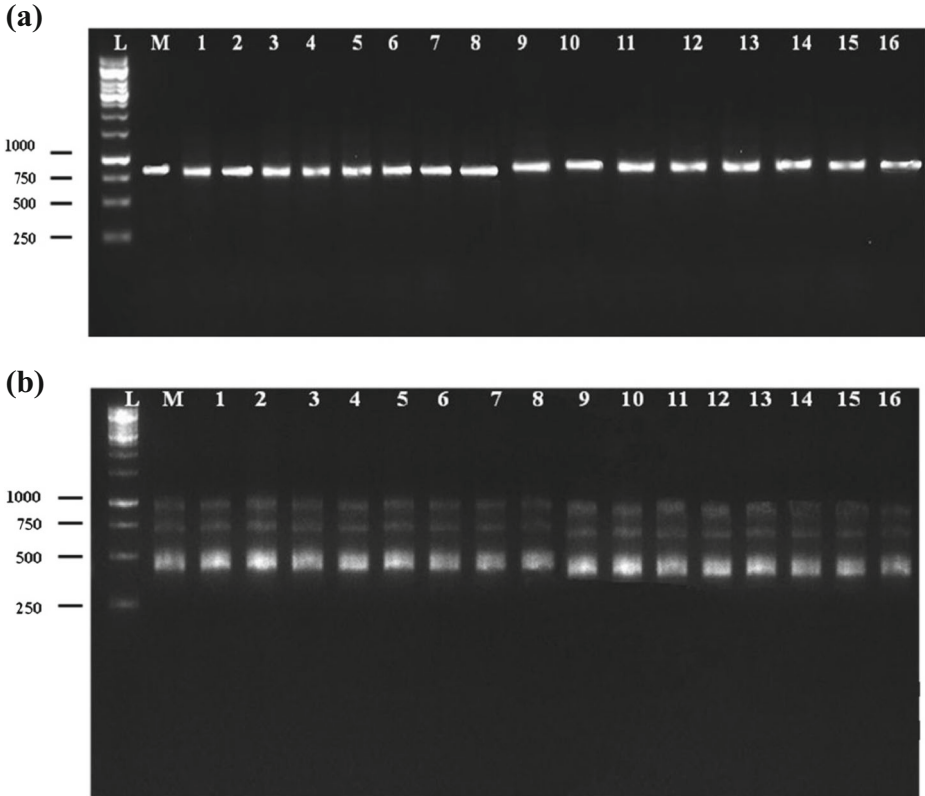


Fig. 5 ISSR amplification pattern obtained with primers **a** UBC-843 and **b** UBC-844 for the mother plant and the randomly selected plants raised from synthetic seeds (1–16) after 90 days of storage under slow growth conditions. Lane L molecular marker, lane M mother plant, lane 1–8 the plants grown from synthetic seeds stored at 4 °C, lane 9–16 the plants grown from synthetic seeds stored at 24 °C

(vegetative) propagules for the preparation of synseeds would be in those cases where somatic embryogenesis is not well established or somatic embryos do not germinate into complete plantlets. In such cases, any explants that are totipotent for organogenesis can be used for encapsulation instead of somatic embryos. Synseeds produced after encapsulating vegetative propagules can be used for cost-effective mass clonal propagation, potential long-term germplasm storage, and delivery of tissue-cultured plants. Encapsulation of nodal segments was influenced by the concentrations of sodium alginate and calcium chloride. The highest plantlet regeneration was achieved with 3.0 % sodium alginate and 100 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. This combination was also found best in several earlier reports [34, 35, 17]. This differential response may be due to a synergistic effect of alginate and calcium concentration. Both sodium alginate and calcium chloride play an important role in complexation and bead hardness [36]. Moreover, the formation of beads was also influenced by treatment duration of calcium chloride. Treatment duration of 15 min was found best in the present study which is in close accordance with some earlier findings [37, 17]. Media strength has significant impact on shoot regrowth from synseeds. The half-strength solid medium was found best for maximum shoot regrowth. Huang and Trueman [35] have also come to the same conclusion that media strength influences shoot regrowth. Regeneration of shoots for mass multiplication

is affected by the presence of growth regulators like cytokinins and auxins in media. BAP is an important growth regulator that facilitates high-frequency regeneration [38, 7], while BAP-supplemented media inhibited root differentiation. This may be due to the role of cytokinins in suppressing root regeneration [39]. NAA is an important auxin, which facilitated rooting in many important plants [40]. A substantial number of micropropagated plants do not survive when transferred from the in vitro condition to the field environment; thus, acclimatization of micropropagated plants is very essential. For short-term conservation of the germplasm, encapsulation technology is widely used in different medicinal [41, 42], horticultural [43, 44], and other valuable [45, 46] plants. The prerequisite for conservation of encapsulated seed is to store the synseeds for a longer duration without affecting their viability. In present study, low temperature was better than culture room temperature for storage and maximum shoot regrowth. Low-temperature storage has been found suitable in several other medicinal plants with maximum recovery of plantlets [47]. This was probably due to low temperature which slows down the metabolic activities of the synseeds; hence, they remained in a quiescent state that is helpful for the preservation of the nutritive reservoir in the synseeds during cold storage [48]. The decline in plant recovery from stored encapsulated nodal segments may be due to oxygen deficiencies in the calcium alginate bead and its rapid drying as well as dehydration of explants [49]. The most desirable feature of encapsulated propagules is their capability to retain viability after storage for a reasonable period required for exchange of germplasm between laboratories [50]. Plants regenerated from nodal segments are also prone to genetic variation [51–54]. Therefore, genetic variability may also be expected from plants regenerated from explants having preexisting meristems like node and shoot tip. The genetic fingerprints of regenerated plants from synseeds after 90 days of storage were compared with those of the mother plant using RAPD and ISSR markers. Genetic fidelity analysis confirmed that plants regenerated from encapsulated nodal segments were genetically similar to the donor mother plant. RAPD and ISSR markers were widely used by several workers [55, 25, 26] for establishment of genetic stability of synseed-derived regenerated plants after in vitro storage. These markers were chosen because of their simplicity and cost-effectiveness. They amplify different regions of the genome, providing a broad analysis of genetic stability or variation in plants.

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