Hortic. Environ. Biotechnol. 57(1):88-96. 2016.
DOI 10.1007/s13580-016-0096-7

ISSN (print) : 2211-3452
ISSN (online) : 2211-3460

Research Report

Germination and Storability of Calcium-Alginate Coated Somatic Embryos of Mango Ginger (*Curcuma amada* Roxb.)

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Received June 17, 2015 / Revised September 28, 2015 / Accepted January 28, 2016

Abstract. We developed a protocol to produce synthetic seeds of mango ginger (*Curcuma amada* Roxb.) for large-scale propagation, storage, and germplasm exchange. The seeds were produced by encapsulating somatic embryos in a calcium–alginate matrix. Embryogenic callus was induced from leaf sheath explants on 0.8% agar solidified with full-strength Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose, 2.0 $mg \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 $mg \cdot L^{-1}$ 6-benzyladenine (BA). Somatic embryos were induced by transferring the calli to 1/2 MS liquid medium supplemented with 3% (w/v) sucrose and 0.3 $mg \cdot L^{-1}$ BA. A synthetic seed coat for the somatic embryos that is uniform in size and shape was produced by incubating the embryos in 3% (w/v) sodium alginate in 1/2 MS liquid medium and exposure to 100 mM calcium chloride ($CaCl_2.2H_2O$) for 15 min. The highest germination percentage of synthetic seeds (91.66%) was achieved on 1/2 MS solid medium supplemented with 3% (w/v) sucrose and 0.25 $mg \cdot L^{-1}$ gibberellic acid (GA_3) in darkness (24 h) for 35 days. Sucrose-dehydrated synthetic seeds showed better storability than fresh seeds. Synthetic seeds that were dehydrated in 8.55% (w/v) sucrose solution and stored at 4°C showed germination percentages of 88.10% after 30 days of storage and 54.16% after 120 days of storage. Plantlets were successfully acclimatized to ex vitro conditions (survival rate, 82.66%) and showed normal phenotypes. In a random amplified polymorphic DNA (RAPD) analysis, a monomorphic banding profile was obtained from the plants derived from synthetic seeds that had been stored at 4°C for 120 days. This confirmed the genetic fidelity of the plants derived from the stored artificial seeds.

***Additional key words*:** acclimatization, dehydration, germination, RAPD analysis, sucrose, synthetic seeds

Introduction

Mango ginger (*Curcuma amada* Roxb.) belongs to the family Zingiberaceae. It is a unique spice that resembles ginger (*Zingiber officinale*), and it is the second most widely cultivated *Curcuma* species after turmeric (*Curcuma longa*) because of its high curcumin content (Syamkumar and Sasikumar, 2007). The rhizome, which is used to make pickles, has a mango-like flavor due to the presence of car-3-ene and cis-ocimene compounds (Gholap and Bandyopadhyay, 1984). There are 68 volatile aromatic compounds and more than 130 chemical constituents in mango ginger (Dutt and Tayal, 1941; Gholap and Bandyopadhyay, 1984; Rao et al., 1989; Choudhury et al., 1996; Srivastava et al., 2001; Singh et al., 2003; Mustafa et al., 2005; Jatoi et al., 2007). The starch in mango ginger rhizomes has metabolic advantages when consumed by humans, because of its low solubility and high amylose content (Policegoudra and Aradhya, 2008). When added to a basal diet, a curcumin-free fraction prepared from mango ginger was shown to lower liver cholesterol in animals (Srinivasan et al., 2008). In addition, extracts from mango ginger rhizomes have been shown to have various biological activities such as antimicrobial, antioxidant, anti-cancer, anti-inflammatory, antidepressant, antitubercular, and platelet aggregation inhibitory activities (Policegoudra et al., 2010). Antibacterial compounds, such as difurocumenonol, amadannulen, and amadaldehyde (Policegoudra et al., 2007), and potential anti-tubercular agents, such as labdane-type diterpene dialdehydes, have also been isolated from the rhizome of mango ginger (Singh et al., 2010).

Mango ginger is a sterile triploid plant, and the rhizomes are used for vegetative propagation and sold for consumption.

However, the rhizomes are slow to multiply, and highly susceptible to physiological and temperature stress during storage (Policegoudra and Aradhya, 2007). Therefore, large-scale production methods are required to meet the increasing demand for mango ginger. In this regard, tissue culture techniques that produce somatic embryos could be used to produce seedlings. Somatic embryos are potentially easier to handle than rhizomes, because they are relatively small and uniform in size. Additionally, somatic embryos have the potential for long-term storage through cryopreservation or desiccation (Cervelli and Senaratna, 1995). The use of somatic embryos would allow flexibility in scheduling production and transport without loss of viability, and would maintain the clonal properties of the regenerated plantlets (Ghosh and Sen, 1994; Cervelli and Senaratna, 1995). Therefore, somatic embryos have potential applications in large-scale production of mango ginger plants.

Synthetic or artificial seeds (or synseeds) have been described as "artificially encapsulated somatic embryos, shoots or other tissues which can be used for sowing under in vitro or ex vitro conditions'' (Aitken-Christie et al., 1995; Germanà et al., 2011). Synthetic seed production technology that uses alginate encapsulation is considered an efficient choice for both propagation and germplasm storage of elite genotypes (Ara et al., 1999). The alginate coat shields the encapsulated explants from physical and environmental injury, reduces dehydration, and provides mechanical pressure to physically support the explants inside the gel matrix during storage (Ara et al., 2000). In addition, artificial seeds are a low-cost propagation method (Ghosh and Sen, 1994; Saiprasad and Polisetty, 2003). The success of synthetic seed technology depends on the quality of propagules. Somatic embryos represent a uniform developmental stage with reversible arrested growth, and show high rates of conversion upon planting (Pinto et al., 2008; Cheruvathur et al., 2013b). However, the development of an efficient somatic embryogenesis system is a major prerequisite for the successful production of synthetic seeds (Bapat and Mhatre, 2005). Once a reliable somatic embryo induction system is developed, the entire artificial seed production process can be automated (Ibaraki and Kurata, 2001). However, the most desirable characteristic of the encapsulated explants is their ability to regrow and form normal plants (Micheli et al., 2007; Parveen and Shahzad, 2014). It is important to optimize methods for the storage, germplasm exchange, and regrowth of encapsulated explants (Sundararaj et al., 2010). In addition, the molecular status of plants derived from synthetic seeds must be determined for successful germplasm exchange; this status can be examined using RAPD (Random amplified polymorphic DNA) markers. RAPD markers have been widely used because of their simplicity, low cost, and wide availability, and they provide an easy method to detect clonal integrity (Prakash et al., 2004; Agnihotri et al., 2009; Mohanty and Das, 2013).

Synseed technology has been used in various agricultural crops and in some ornamental and medicinal plant species. To date, synthetic seeds containing auxiliary buds have been produced for several members of the ginger family, including *Z. officinale* (Sharma et al., 1994; Sundararaj et al., 2010), *C. longa* (Gayatri et al., 2005), and *C. amada* (Banerjee et al., 2012). However, there are no reports on the use of somatic embryos of the ginger family in synthetic seeds. In a previous study, we induced somatic embryos in a cell suspension culture of mango ginger (Soundar Raju et al., 2013). The aim of the present study was to develop a method for the production of synthetic seed by embedding somatic embryos in a calcium–alginate matrix and for germination of these embryos after storage. We optimized various factors in these processes, including medium strength, sucrose concentration during dehydration, and light/dark conditions. We evaluated the effects of these factors on the storability of synthetic seeds. Finally, we confirmed the genetic fidelity of the germinated plants through RAPD analysis.

Materials and Methods

Plant Material and Somatic Embryogenesis

C. amada plants were collected from Malappuram, Kerala, India. Pieces of rhizome with buds were established in vitro and used as a source of explants. The culture procedures and media were similar to those described by Soundar Raju et al. (2013). Embryogenic callus was induced from leaf sheath explants (~1.5 cm long) of 3-month-old in vitro-grown plantlets. The tender leaf tips were avoided and peripheral leaf sheath explants were cultured for 21 days on 0.8% (w/v) agar solidified in full strength MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, 2.0 $mg \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 $mg \cdot L^{-1}$ 6-benzyladenine (BA). Embryogenic calli were formed during this period (Fig. 1A). Somatic embryo induction and maturation were achieved by transferring the embryogenic callus to 1/2 MS liquid medium supplemented with 3% (w/v) sucrose and 0.3 $mg \cdot L^{-1}$ BA (Fig. 1B). The cultures were incubated in an Erlenmeyer flask with continuous shaking at 110 rpm. The embryos were allowed to mature for 35 days, and were transferred to fresh medium every 10 days. All chemicals were purchased from HiMedia Pvt. Ltd. (HiMedia, Mumbai, India). The pH of the medium was adjusted to 5.6 ± 0.2. All media and equipment were sterilized by autoclaving at 121°C and 104 kPa for 15 min. All cultures were maintained at 25 ± 2°C, under a 16 h light/8 h dark photoperiod and a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ produced by white fluorescent lamps (Philips, Kolkata, India).

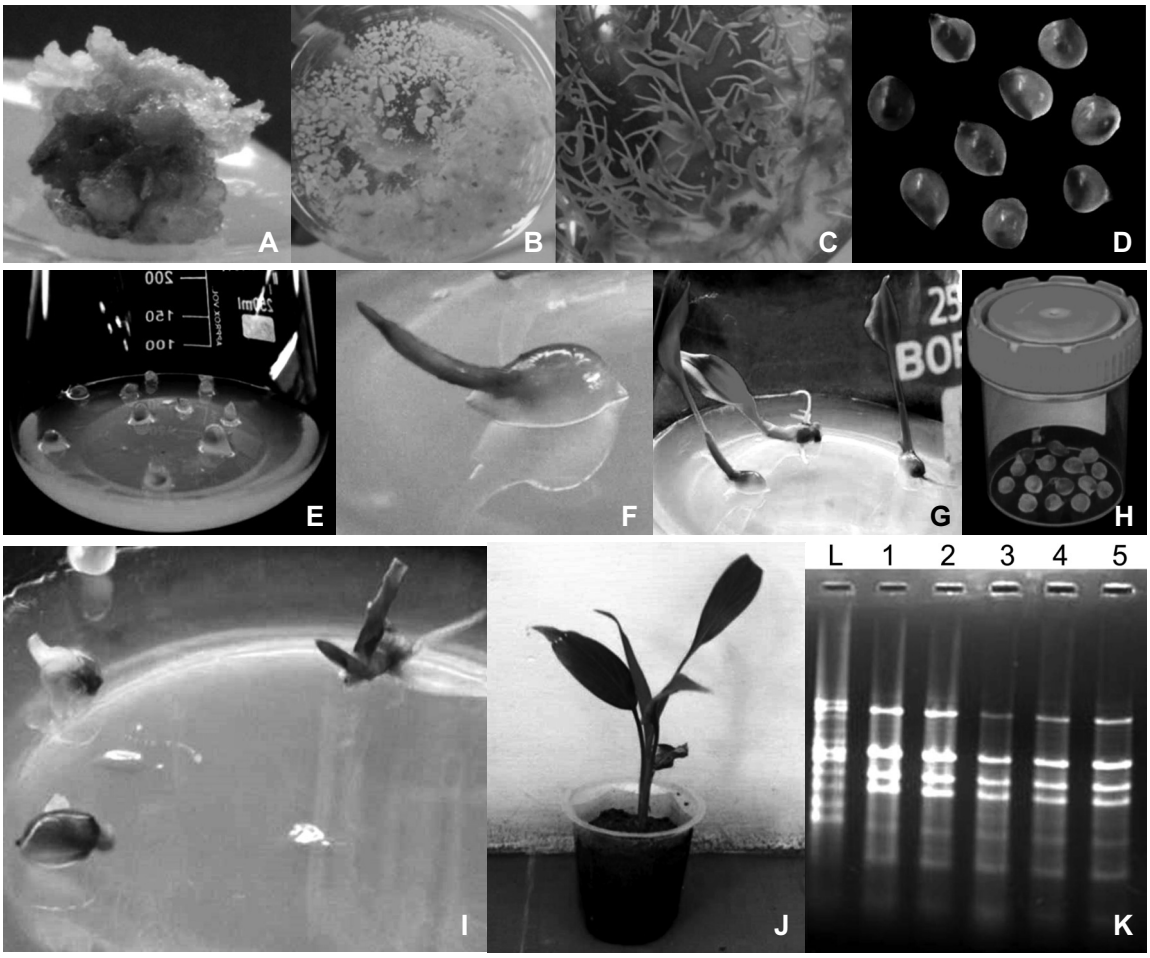

Fig. 1. Somatic embryogenesis and synthetic seed production in *C. amada*: A, Embryogenic calli were induced on full-strength MS solid medium supplemented with 3% (w/v) sucrose, 2.0 mg·L^{-1} 2,4-D and 0.5 mg·L^{-1} BA; B, Somatic embryos were induced by transferring embryogenic calli to 1/2 MS liquid medium supplemented with 3% (w/v) sucrose and 0.3 mg·L^{-1} BA; C, Mature somatic embryos; D, Synthetic seeds produced by encapsulating somatic embryos in 3% (w/v) sodium alginate and exposure to 100 mM $CaCl_2.2H_2O$ for 15 min; E, Somatic embryos in calcium–alginate matrix turned greenish-white before germinating on 1/2 MS solid medium supplemented with 3% (w/v) sucrose and 0.25 mg·L^{-1} GA_3 in darkness (24 h); F, Germinated synthetic seeds; G, Plantlets derived from somatic embryos encapsulated in synthetic seeds; H, Sucrose-dehydrated synthetic seeds stored in sample container; I, Germination of synthetic seeds dehydrated in 8.55% (w/v) sucrose after 120 days of storage at 4°C; J, Ex vitro acclimatized plant (derived from synthetic seed) growing in a mixture of sand, soil, and vermiculite (1:2:1); K, RAPD amplification patterns produced using OPN06 primer: 1, donor mother plant; 2, plant derived from somatic embryo; 3–5, randomly selected synthetic seed-derived plants after 120 days storage at 4°C. (Ladder (L) = 30000 bp).

Synthetic Seed Production

Mature somatic embryos (0.5 cm in length) were transferred to 1/2 MS liquid medium containing 1, 2, 3, or 4% (w/v) sodium alginate. Subsequently, the sodium alginate solution with somatic embryos was pipetted with a 1 mL micropipette (tip diameter, 0.5 cm), and the solution with a single embryo was dropped into a sterile 100 mM $CaCl_2.2H_2O$ solution. The alginate beads were exposed to the solution for 5, 10, 15, 20, 25, or 30 min, with occasional gentle agitation for successful encapsulation of the matrix. The calcium-alginate beads were washed with sterile distilled water and blotted on filter paper to remove traces of $CaCl_2.2H_2O$. The moisture content of calcium-alginate beads was estimated according to Cordeiro et al. (2014) and transferred onto 1/2 MS solid medium containing 1.5% (w/v) sucrose to germinate in the dark, as described previously (Soundar Raju et al., 2013). Before encapsulation, sodium alginate/$CaCl_2.2H_2O$ solutions were autoclaved at 121°C for 20 min.

Optimization of Media Components and Incubation Conditions for Germination

To assess the effects of GA_3 and photoperiod on germination, the beads were cultured on 1/2 MS solid medium supplemented with 1.5% (w/v) sucrose and different concentrations of GA_3 (0, 0.25, 0.5, 0.75, and 1.0 mg·L^{-1}). The cultures were incubated either under partial illumination (16 h/8 h, light/dark) or under complete darkness (24 h) for 35 days. In experiments to test the effect of the strength of the

medium (1/4, 1/2, 3/4, and full strength MS) and the sucrose concentration [1, 2, 3, 4, 5, 6, and 7% (w/v)] on artificial seed germination, the medium contained 0.25 $mg \cdot L^{-1}$ GA_3, and the seeds were germinated in darkness.

Storability of Synthetic Seeds

Synthetic seeds were dehydrated in sucrose solutions (8.55% or 17.11%, w/v) for 24 h with continuous orbital shaking at 70 rpm. Fresh (non-dehydrated) synthetic seeds were used as the control. All experiments were performed under sterile conditions. The dehydrated seeds were transferred to 1/2 MS solid medium containing 3% (w/v) sucrose and 0.25 $mg \cdot L^{-1}$ GA_3 to germinate in the dark. To test the storability of encapsulated somatic embryos, both sucrose-dehydrated or non-dehydrated beads were stored at 4°C or 25°C in sealed 50 mL sample containers (15 embryos per container; Tarsons, Kolkata, India) for 30, 60, 90, and 120 days. Finally, the synthetic seeds were transferred onto the medium described above to germinate in the dark.

Acclimatization

Three weeks after germination, the plantlets were transferred to 16 h light/8 h dark conditions for further growth and to induce chlorophyll accumulation. Plantlets with 3-4 leaves and 4-5 roots were transferred to plastic cups containing a mixture of autoclaved sand, soil, and vermiculate (1:2:1). The plantlets were maintained at 25 ± 2°C under a 16 h light/8 h dark photoperiod for 10 days before being transferred to ex vitro conditions. The survival rate was calculated after 30 days of acclimatization.

RAPD Analysis to Assess Genetic Fidelity

To analyze genetic stability, young leaves were selected randomly from plants derived from synthetic seeds that were stored for 120 days and acclimatized for 30 days. Field-grown plants and somatic embryo-derived plants were used as controls. Total genomic DNA was isolated from young leaves using the CTAB procedure (Rogers and Benedich, 1994; Prakash et al., 2004). The DNA yield and purity were determined by calculating the OD_{260} nm/OD_{280} nm ratio using a UV-Vis spectrophotometer (V-550, Jasco, Japan) and also by agarose gel electrophoresis (Chromous Biotech, Bangalore, India). The gel contained 0.8% (w/v) agarose dissolved in 1 × TBE buffer, and DNA fragments were stained with ethidium bromide and visualized by ultraviolet light (UV). Polymerase chain reactions (PCRs) were carried out in a Thermal Cycler (Eppendorf, USA Scientific inc.) using 15 RAPD Operon primers (Aslam et al., 2010). Each 25 μL reaction mixture contained 50 ng template DNA, 200 μM dNTP mix, 1 unit Taq DNA polymerase, 1 × PCR buffer containing Tris and 15 mM $MgCl_2$ (Bangalore Genei, Bangalore, India), and 10 pM primer. The cycling conditions were as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, primer annealing at 37°C for 30 s, and extension at 72°C for 2 min, followed by final extension at 72°C for 7 min. The amplified products were resolved on a 1.5% agarose gel, detected with a UV transilluminator, and documented with gel documentation software (Gelstan, Mediccare, India).

Statistical Analysis

The experimental data were collected from three independent experiments, each consisting of a minimum of 24 synthetic seeds prepared from 35-day-old mature embryos. Data were subjected to one-way ANOVA followed by statistical significance tests. Reported values are mean ± standard error (SE). Mean separations were analyzed using Duncan's multiple range test at a significance level of $p < 0.05$ using SPSS software (ver. 19; IBM, Armonk, NY, USA).

Results

Synthetic Seed Production

In the present study, mature somatic embryos (obtained from embryogenic callus in a cell suspension culture) were used to produce synthetic seeds (Fig. 1C). We determined the effects of different concentrations of sodium alginate and different exposure times to 100 mM $CaCl_2.2H_2O$ on the quality of synthetic seeds (Fig. 1D and 2). Initially, encapsulated somatic embryos turned greenish-white before germinating and forming roots (Fig. 1E and 1F). The size and shape of the beads was uniform when the 3% (w/v) sodium alginate solution was exposed to 100 mM $CaCl_2.2H_2O$ solution for 15 min. The germination percentage of these artificial seeds was 68.05%. The seeds produced by using 4% (w/v) sodium alginate exposed to 100 mM $CaCl_2.2H_2O$ for 15 min formed the hardest coat and showed a germination percentage of 51.38%. At lower concentrations of sodium alginate [1 and 2% (w/v)] and exposure to 100 mM $CaCl_2.2H_2O$, the beads were non-uniform and fragile, probably due to high moisture content (data not shown). The germination percentages of these seeds were low. Sodium alginate at 3 or 4% (w/v) with either shorter (5 or 10 min) or longer exposure times (20, 25, or 30 min) to 100 mM $CaCl_2.2H_2O$ also resulted in poor germination percentages.

Optimization of Media Components and Incubation Conditions for Germination

The germination rate of synthetic seed varied depending on the GA_3 concentration in the medium and on the light conditions [either partial illumination (16 h/8 h, light/dark) or complete darkness (24 h)]. The maximum germination

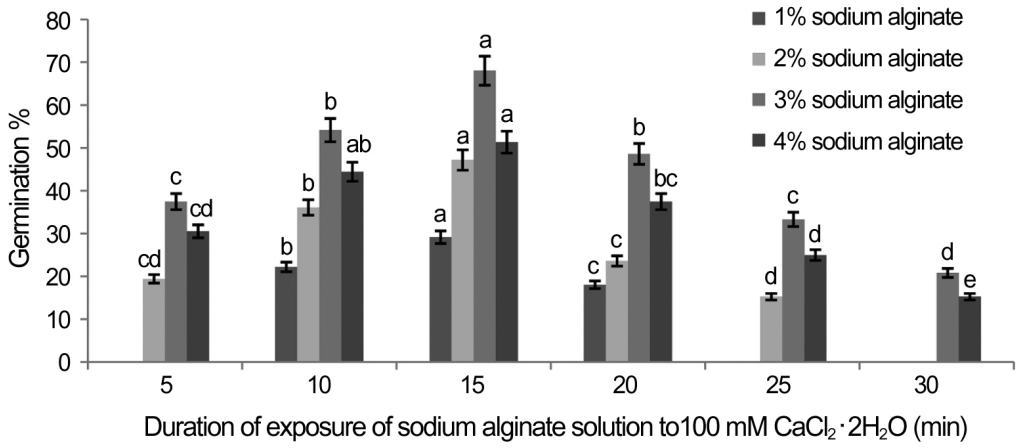

Fig. 2. Effects of different concentrations of sodium alginate and different exposure times to 100 mM $CaCl_2.2H_2O$ on germination of synthetic seeds. Seeds were germinated on 1/2 MS solid medium supplemented with 1.5% (w/v) sucrose in darkness. Values are mean ± SE of three independent experiments ($n \geq 24$ synthetic seeds). Data were recorded after 35 days of culture. Different letters in each column indicate significant difference at $p < 0.05$ (Duncan's multiple range test).

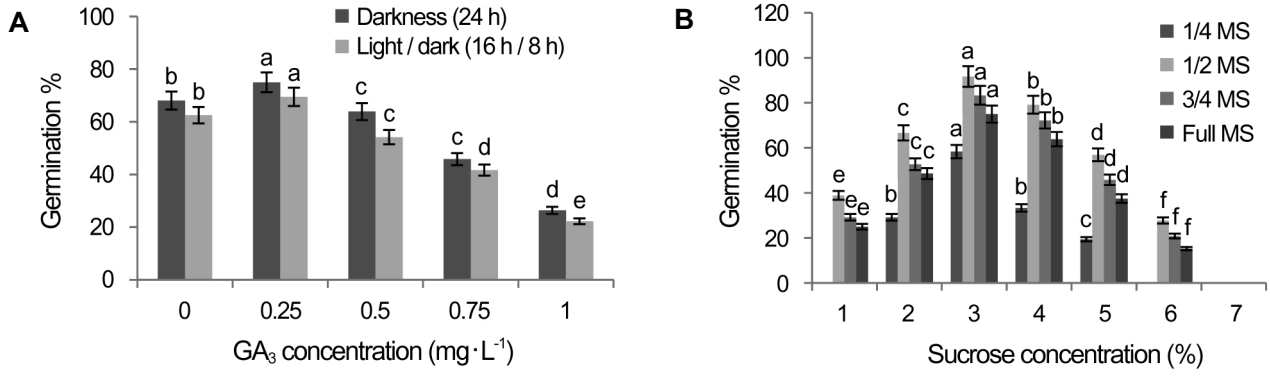

Fig. 3. Germination percentages of synthetic seeds: A, Seeds were germinated on 1/2 MS solid medium supplemented with 1.5% (w/v) sucrose and different concentrations of GA_3 in the light/dark (16 h/8 h) or darkness (24 h); B, Seeds were germinated on medium containing MS salts at different strengths, sucrose at different concentrations, and 0.25 mg·L^{-1} GA_3 in darkness (24 h). Values are mean ± SE of three independent experiments (n ≥ 24 synthetic seeds). Data were recorded after 35 days of culture. Mean values showed by different letters in each column are significantly different at $p < 0.05$ (Duncan's multiple range test).

percentage of 74.99% was achieved on medium containing GA_3 (0.25 mg·L^{-1}) in darkness (24 h). The germination percentage on medium without GA_3 was 68.05% after dark incubation (Fig. 3A). Germination was also inhibited at higher GA_3 concentrations. Seeds incubated in the dark showed higher germination percentages than seeds incubated under partial illumination. The germination of the dormant somatic embryos was also affected by the sucrose concentration and the strength of the MS medium. Among the various media strengths and sucrose concentrations, an optimum percentage of germination (91.66%) was obtained on 1/2 MS solid medium containing 3% (w/v) sucrose and 0.25 mg·L^{-1} GA_3 under dark conditions (Fig. 1G and 3B). The germination percentage was lower at other media strengths and other concentrations of sucrose (Fig. 3B). None of the artificial seeds germinated on medium containing 7% (w/v) sucrose.

Storability of Synthetic Seeds

In a preliminary experiment, the germination percentage was 91.66% in the control but lower (86.11%) after dehydration in 8.55% (w/v) sucrose. None of the seeds that were dehydrated in 17.11% (w/v) sucrose germinated. In the present study, the sucrose-dehydrated seeds showed a higher germination rate than that of control seeds after storage (Fig. 1H and 1I; Table 1). Therefore, the germination potential of encapsulated somatic embryos after storage at 4 and 25°C was evaluated. After 30 days of storage at 4°C, control seeds showed a germination rate of 77.77%, while sucrose-dehydrated seeds showed a germination rate of 88.10%. After 30 days of storage at 25°C, 66.66% of control seeds germinated and 72.22% of sucrose-dehydrated seeds germinated. With increased storage time, the germination rate decreased for all synthetic seeds. After 120 days of storage,

Table 1. Germination of synthetic seeds on 1/2 MS solid medium supplemented with 3% (w/v) sucrose and 0.25 mg·L^{-1} GA_3 in darkness (24 h), after storage at 4 and 25°C

Storage temperature (°C)	Storage time (days)	Germination (%)	
		Fresh seeds (control)	Sucrose-dehydrated seeds*
4	0	91.66 ± 3.67^a	86.11 ± 2.40^a
	30	77.77 ± 3.67^b	88.10 ± 3.67^a
	60	58.33 ± 2.40^c	73.61 ± 1.39^b
	90	20.83 ± 2.40^d	61.10 ± 3.67^c
	120	11.11 ± 1.39^e	54.16 ± 2.40^c
25			
	30	66.66 ± 4.81^a	72.22 ± 2.78^a
	60	33.33 ± 4.81^b	56.88 ± 3.63^b
	90	12.49 ± 2.40^c	37.49 ± 4.81^c
	120	0.0	0.0

Values are mean ± SE of three independent experiments (n ≥ 24 synthetic seeds). Data were recorded after 35 days of culture. Mean values followed by different letters in each column are significantly different at $p < 0.05$ (Duncan's multiple range test). *Seeds were dehydrated in 8.55% (w/v) sucrose solution.

the sucrose-dehydrated synthetic seeds that were stored at 4°C showed a germination rate of 54.16%, whereas none of those that were stored at 25°C germinated. Compared with control seeds, the sucrose-dehydrated synthetic seeds showed much higher germination percentages after 120 days of storage at 4°C. Both control and sucrose-dehydrated seeds showed maximum survival rates at 25°C for a period of 90 days. Together, these results show that sucrose-dehydrated synthetic seeds have the highest survival rates when stored at 4°C.

RAPD Analysis to Assess Genetic Fidelity

Plantlets derived from synthetic seeds were successfully acclimatized and showed a survival rate of 82.66% after 30 days of acclimatization (Fig. 1J). From the 15 primers used in the RAPD analysis, 5 primers (OPA-02, OPA-06, OPD-14, OPM-16, and OPN-06) produced bands that were consistently reproducible with a total of 30 bands and an average of 6.0 bands per primer. The RAPD patterns were compared between randomly selected plants derived from stored artificial seeds and control plants (donor mother plants and plants derived directly from somatic embryos). The sizes of the DNA fragments that were produced using these primers ranged from 100 bp to 2500 bp. As a representative result, Figure 1K shows the monomorphic band pattern produced in the RAPD analysis using the primer OPN06. Together, the amplification profiles confirmed the genetic similarity between control plants and those derived from synthetic seeds stored for 120 days at 4°C.

Discussion

Synthetic seed technology can enable rapid plant multiplication via somatic embryogenesis (Castillo et al., 1998). Synthetic seeds and somatic embryos have many advantages over organogenesis for propagation: they are easier to handle, can be stored for longer periods, have the potential for scale-up, and are cost-effective to produce and propagate (Ghosh and Sen, 1994). The potential to automate the entire process provides another substantial advantage, because high volumes will be required for commercial applications (Ziv, 1995).

In the production of synthetic seeds, the texture of beads is strongly influenced by the gel matrix (sodium alginate) and the complexing agent ($CaCl_2.2H_2O$). Sodium alginate plays an important role in formation of the artificial endosperm that protects the propagule. It has been extensively used in synthetic seeds because of its low cost, gelling properties, low toxicity, sufficient rigidity, and ease of handling (Redenbaugh et al., 1987; Bapat and Mhatre, 2005). To produce uniform beads with optimal hardness, the conditions for bead hardening must be optimized to allow for appropriate ion exchange between the Na^+ in sodium alginate and Ca^{2+} in $CaCl_2.2H_2O$ solution. For this purpose, the concentrations of both solutions and the complexing time must be optimized (Saiprasad, 2001; Singh et al., 2009).

In this study, lower concentrations [1 and 2% (w/v)] of sodium alginate resulted in the formation of soft and fragile beads that were difficult to handle. The use of 4% (w/v) sodium alginate resulted in the formation of isodiametric beads that were very hard, which considerably delayed germination. The use of 3% (w/v) sodium alginate resulted in firm, good-quality beads upon exposure to 100 mM $CaCl_2.2H_2O$. The seeds produced using these reagents showed a germination rate of 68.05%. Similar results have been reported in encapsulated somatic embryo propagules of *Dendrobium densiflorum* (Mohanty and Das, 2013), and *Rinacanthus*

nasutus (Cheruvathur et al., 2013a). In contrast, 4% (w/v) sodium alginate with 100 mM $CaCl_2.2H_2O$ showed uniformly sized clear beads in encapsulated microshoots propagules of *Z. officinale* (Sundararaj et al., 2010) and nodal segments of *Decalepis hamiltonii* (Sharma and Shahzad, 2012).

In this study, the duration of exposure of sodium alginate to $CaCl_2.2H_2O$ significantly affected the germination rate of encapsulated somatic embryos of *C. amada*. The highest germination percentage was achieved using beads produced with 3% (w/v) sodium alginate and exposed to 100 mM $CaCl_2.2H_2O$ for 15 min. Exposure to 100 mM $CaCl_2.2H_2O$ for 20-30 min resulted in very hard beads and low germination rates. These results are consistent with those reported by Malabadi and van Staden (2005) for *Pinus patula*.

The most desirable property of synthetic seeds is their ability to remain viable for germination and regrowth (Adriani et al., 2000; Micheli et al., 2007; Parveen and Shahzad, 2014). In the present study, firm and clear beads obtained using 3% (w/v) sodium alginate and 100 mM $CaCl_2.2H_2O$ were transferred to 1/2 MS medium with or without GA_3, and were germinated under illumination (16 h/8 h, light/dark) or under complete darkness (24 h). The synthetic seeds that were cultured on 1/2 MS medium without GA_3 showed a germination rate of 68.05% after 35 days of culture. The presence of GA_3 resulted in a germination rate of 74.99% after 35 days of culture. Other studies have reported that GA_3 is essential for germination of synthetic seeds, for example those of *Eleutherococcus senticosus* (Choi and Jeong, 2002) and *R. nasutus* (Cheruvathur et al., 2013a). Incubation under complete darkness (24 h) enhanced the germination of somatic embryos in the studies by Garcia-Martin et al. (2001) and Soundar Raju et al. (2014). Similarly, in this study the germination percentages were higher in the complete darkness (24 h) than under illumination (16 h/8 h, light/dark).

Several studies have shown that sucrose affects the morphogenetic responses of different plant species. Sucrose is an important carbon source in tissue culture medium, and also functions as an osmotic compound (Sujatha and Ranjitha Kumari, 2008). According to Bozena and Szezebra (1991), sucrose is commonly used in tissue culture media because of its efficient uptake across the plasma membrane. In the present study, the germination of the synthetic seeds was affected by the sucrose concentration in the media. Among the different concentrations tested, 3% (w/v) sucrose was optimal for germination. Similar results have been reported for *Spartina alterniflora* (Utomo et al., 2008).

Nutrient concentrations also play an important role in synthetic seed germination and growth (Gantait and Sinniah, 2013; Mohanty and Das, 2013). We tested MS medium at four concentrations (1/4, 1/2, 3/4, and full strength) and observed that the highest germination percentages were achieved on 1/2 strength MS medium containing 0.25 $mg \cdot L^{-1}$ GA_3 and 3% (w/v) sucrose. Similar results were reported for *E. senticosus* (Choi and Jeong, 2002). This finding suggests that the concentration of MS nutrients is an important factor in the growth of shoots and roots from the encapsulated somatic embryos.

Sugar accumulation in plant tissues is a well-known strategy for tolerance to stress, including dehydration stress. The accumulated sugars stabilize proteins and membrane bilayers (Zhu et al., 2006). Maintaining the viability of synthetic seeds after a long period of storage is an important prerequisite in the synthetic seed industry (Naik and Chand, 2006; Micheli et al., 2007). In the present study, dehydration of synthetic seeds in 8.55% (w/v) sucrose for 24 h provided sufficient sucrose to sustain the growth of synthetic seeds during germination and development. No germination was observed after dehydration of synthetic seeds using 17.11% (w/v) sucrose. After storage, germination rates of sucrose-dehydrated seeds were much higher than those of fresh seeds. Similar results have been reported for *Z. officinale* (Sundararaj et al., 2010) and *Mandevilla moricandiana* (Cordeiro et al., 2014). When the encapsulated embryos were stored at two different temperatures (4 and 25°C), those stored at 4°C for 120 days showed a germination percentage of 54.16%, but those stored at 25°C did not germinate. Similar results have been reported for artificial seeds of *Clitoria ternatea* (Kumar and Thomas, 2012), which remained viable even after 5 months of storage at 4°C.

The genetic fidelity of micropropagated plants is extremely important for commercial applications (Paul et al., 2010). RAPDs are simple, cost-effective molecular markers, and have been used to analyze the genetic fidelity of several plant species that are cultured in vitro (Gupta and Roy, 2002; Panda et al., 2007; Sun et al., 2009; Kwak et al., 2009; Banerjee et al., 2012; Rad et al., 2014). Factors that contribute to genetic instability in vitro include chromosomal arrangements, single gene mutations, pre-existing DNA variability, sub- and supra-optimal concentrations of plant growth regulators, especially synthetic ones, activation of retrotransposons, the length of the culture period, and the regeneration method (Martins et al., 2004). In the RAPD analyses in this study, no polymorphisms or changes in the amplified DNAs were detected between plants derived from synthetic seeds and control plants. This result confirmed that the storage of synthetic seeds did not affect their genetic integrity (Mohanty and Das, 2013).

In conclusion, the present study identifies a protocol for successful establishment of synthetic seeds from somatic embryos of *C. amada*. The results showed that uniform synthetic seeds can be formed in 3% (w/v) sodium alginate complexation with 100 mM $CaCl_2.2H_2O$. Germination of these synthetic seeds was optimal (91.66%), when somatic embryo propagules were cultured on 1/2 strength solid MS

medium supplemented with 3% (w/v) sucrose and 0.25 $mg \cdot L^{-1}$ GA_3 under dark conditions (24 h). Synthetic seeds that were dehydrated in 8.55% (w/v) sucrose solution and stored at 4°C remained viable for up to 120 days. RAPD analysis confirmed that this protocol does not induce genetic changes in the synthetic seeds that were derived from *C. amada* plants. This simple and efficient method for the production of synthetic seeds could be used for storage and exchange of valuable germplasm of *C. amada*.

Acknowledgment: The authors thank DST, Government of India, for providing facilities through the FIST program and the University Grants Commission, New Delhi, for its support through the "College with Potential for Excellence" program.

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