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Regeneration of *Renanthera imschootiana* Rolfe using Synthetic Seeds

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Abstract: Synthetic *'*seeds*'* were produced from encapsulated protocorm-like bodies (PLBs) obtained from axenic leaf explants of *Renanthera imschootiana* Rolfe. The physical characteristics (size, shape, texture etc.) of the 'seeds' varied with the concentration of sodium alginate, calcium chloride, and the subsequent complexation period. The 'seeds' were best formed (spherical and firm) using 4% sodium alginate and 75 mM calcium chloride and a subsequent complexation period of 30 min. The conversion frequency of 'seeds' was markedly influenced by their shape and texture. More or less firm beads with spherical outline converted with 100% frequency and the soft or very hard ones showed a poor conversion frequency. The freshly formed seeds germinated readily with cent per cent frequency. The conversion frequency was detrimentally affected upon storage; it was reduced to 60% after 20 days, 40% after 40 days, 20% after 60 days, and 0% after 80 days of storage at 25°C. However, when stored at 4°C, the synthetic 'seeds' retained cent per cent convertibility up to 40 days but subsequently, their conversion frequency decreased inversely with the increase in storage period. Nearly 40% 'seeds' lost viability after 60 days and only 20% responded to conversion after 80 days. The convertibility was completely lost after 100 days. The findings suggest that the encapsulation method for PLB obtained from leaf explants of *R. imschootiana* provides a useful alternative tool for conservation of this endangered species.

Keywords: Orchids, *Renanthera*, synthetic seeds, protocorm-like bodies, regeneration.

INTRODUCTION

Despite the rapid development in tissue culture technology, attempts to formulate an appropriate delivery system for the micropropagants have remained meager. In fact, an imbalance between the efficiency of in vitro propagation and the delivery of regenerants poses certain limitations on the practical application of tissue culture technologies. In this connection, the utility of encapsulated somatic embryos (synthetic 'seeds') as an efficient delivery system, first proposed by Murashige (1978), is being increasingly realized. The synthetic 'seed' technology combines the advantages of clonal propagation with low-cost, high-volume capabilities of seed propagation. Its other advantages include: ease of handling, economy of space, time, and medium during storage; and direct planting of propagules into the greenhouse or the field obviating the need of acclimatization procedures normally associated with micropropagated plants. The synthetic 'seeds' were first prepared by Redenbaugh et al. (1986) in alfalfa (Medicago sativa L.); since then such 'seeds' have been prepared in a large number of orchids (Sharma et al., 1992; Corrie and Tandon, 1993; Malemnganba et al., 1996; Nayak et al., 1998; Datta et al., 1999; Vij et al., 2000, 2001; Saiprasad and Polisetty, 2003; Sarmah et al., 2010; Gantait et al., 2012; Mahendran, 2014, Siew et al., 2014). Presently, the efficacy of synthetic seeds was successfully tested in *Renanthera imschootiana*.

Renanthera imschootiana Rolfe, popularly known as 'Red Vanda', is a spectacular epiphytic species of orchids distributed in India and Burma. In India, it dwells in sunny places in Assam, Manipur, Nagaland, and Mizoram within an altitudinal range of 1000-1500 m. The species is of great horticultural value and has been extensively collected and used to produce many elite hybrids at interspecific to intergeneric levels. As of now, the species is extremely rare in its natural habitats and figures prominently in Appendix I of CITES; its collection from the wild has been banned through Schedule I of Wildlife Protection Act of the Government of India. The species is also included in the Threatened Plant List of India published by International Union for Conservation of Nature and natural Resources (IUCN) and Red Data Book of Indian Plants (Jain and Sastry, 1984). This communication reports the successful production of synthetic seeds in *Renanthera imschootiana* and utilizing them for short-term germplasm conservation, and as an efficient propagation and delivery system.

MATERIALS AND METHODS

Selection of propagules

Leaf culture derived protocorm-like bodies (PLBs) were multiplied 10% coconut water supplemented Knudson C (1946) medium to obtain their sufficient quantity and then subcultured on basal medium for 4 wks so as to suppress their further cycles of somatic embryogenesis. The propagules measuring 2 mm in diameter were selected with a view to obtaining their synchronous conversion with high frequency. These were mildly dehydrated by placing them (in petriplates) within folds of filter papers in laminar air-flow cabinet for 1 hr. The propagules thus prepared were used as encapsulants.

Encapsulation

The selected and dehydrated propagules were dispersed in sterilized and cooled sodium alginate solution (2-5%; w/v)

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in an appropriate medium (devoid of $CaCl_2.2H_2O$, if any, since different quantities of this compound were added in complexation solution to obtain its most appropriate concentration for desirable 'seed' formation). This suspension was added drop-wise to a magnetically stirred solution of 50-100 mM $CaCl_2.2H_2O$ solution prepared in the same medium containing sucrose (2%). The resultant beads of calcium-alginate were left in $CaCl_2.2H_2O$ bath for 15-45 min to allow complexation (ionic exchange between sodium alginate and calcium chloride) and subsequently washed 2-3 times with sterilized distilled water before storage/sowing. All these operations were carried out under aseptic conditions.

Storage

The 'seeds' were stored in sterilized vessels at 4°C and 25°C, and their convertibility tested for 120 days at 15/20 days' intervals. The convertibility of both freshly formed and stored 'seeds' was also tested on different sowing substrata like liquid medium irrigated sand/potting mix and non-irrigated sand/potting mix.

RESULTS

'Seeds'

The physical characteristics (size, shape, texture etc.) of the 'seeds' varied with the concentration of sodium alginate, calcium chloride, and the subsequent complexation period. The 'seeds' were best formed (spherical and firm) using 4% sodium alginate and 75 mM calcium chloride and a subsequent complexation period of 30 min (Table 1). These were, however, soft, leaky, and irregular in outline when sodium alginate was used at lower concentrations and very hard in texture at higher concentrations.

Conversion

The conversion frequency of 'seeds' was markedly influenced by their shape and texture. More or less firm beads with spherical outline converted with 100% frequency and the soft or very hard ones showed a poor conversion frequency. Consequently, only the former (spherical, firm, and non-leaky) were used to test their amenability to storage.

Effect of temperature and storage

The effect of storage under different temperature regimes was tested on the conversion frequency at 20 days interval. The results are summarized in Table 2 and described briefly as follows.

i) Storage at 25°C

The freshly formed 'seeds' germinated readily with cent per cent frequency; each producing multiple shoots so that the number of plantlets obtained was much larger than the number of 'seeds' sown. The conversion frequency was detrimentally affected upon storage; it was reduced to 60% after 20 days, 40% after 40 days, 20% after 60 days, and 0% after 80 days of storage.

ii) Storage at 4°C

At this temperature, the synthetic 'seeds' retained cent per cent convertibility up to 40 days but subsequently, their conversion frequency decreased inversely with the increase in storage period. Nearly 40% 'seeds' lost viability after 60 days and only 20% responded to conversion after 80 days. The convertibility was completely lost after 100 days.

Effect of sowing substratum

The *in vitro* conversion frequency of 'seeds', stored at 25°C for 20 days was also influenced by the nature of sowing substratum (Table 3). All the 'seeds' germinated when an agarised substratum was used irrespective of the presence of growth adjuncts in the medium. Under *in vitro* conditions, nearly 60% 'seeds' germinated on nutrient irrigated moss. The frequency of conversion was reduced to 50% and organogenetic process was delayed when nutrient irrigated sand was used instead. Elimination of nutrients, in this case, further impaired the conversion frequency to 20%. The nutrient irrigated epiphytic compost (crockings, charcoal, bark), on the other hand, failed to support conversion.

The problem of fungal and bacterial infections during conversion of 'seeds' under greenhouse conditions could be successfully alleviated by using 0.05% w/v each of bavistin and streptomycin in the nutrient mix. However, the use of anti-microbials impaired the conversion frequency of the synthetic 'seeds'. This loss of convertibility could be compensated by using 1 mg/l each of 1-naphthaleneacetic acid (NAA) and 6-benzylaminpurine (BAP) in the nutrient matrix. The plant hormones favoured rapid proliferation of the viable propagules and number of plantlets produced far exceeded the number of 'seeds' sown. The plantlets were rooted on Banana Homogenate (70 g/l) supplemented basal medium, hardened, and transferred to the greenhouse.

Table 1. Effect of different concentrations of sodium alginate and calcium chloride on 'seed' formation in *Renanthera imschootiana*

Concentration of calcium chloride (mM)	Concentration of sodium alginate (%)			
	2	3	4	5
50	+	++	++	++++
75	+	++	+++	++++
100	+	++	++++	+++++

'+' refers to the quality of 'seeds' in terms of spherical shape and firmness; results are based on an average of 25 replicates

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Table 2. Effect of temperature and storage on conversion frequency of synthetic 'seeds' in *Renanthera imschootiana*

Storage period (days)	Conversion frequency (%) after storage at	
	25°C	4°C
0	100	100
20	60	100
40	40	100
60	20	60
80	-	20
100	-	-

Results are based on an average of 25 'seeds' per treatment

Table 3. Effect of sowing substratum on *in vitro* conversion frequency of synthetic 'seeds' in *Renanthera imschootiana* after 20 days of storage at 25°C

Substratum	Conversion frequency (% germination)	Regeneration pathway	Remarks
KC (agarised)	100	PLB-PL	Good conversion frequency, luxuriant growth of plantlets
Moss + KC (liquid)	60	PLB-PL	Organogenesis delayed
Sand	-	-	'Seeds' desiccated
Sand + KC (liquid)	20	PLB-PL	Suppressed rhizogenesis
Epiphytic Compost (dry)	-	-	'Seeds' desiccated

Results are based on an average of 25 replicates; PLB – protocorm-like body; PL - plantlet

DISCUSSION

Presently, synthetic 'seeds' were successfully prepared in *Renanthera imschootiana* using PLBs as encapsulants. A mild dehydration of the PLBs proved useful for enhancing their tolerance against encapsulation and conversion related stresses, as also emphasized earlier by Sanada *et al*. (1993). Incidentally, Kishi and Takagi (1997) reported that gradual desiccation treatment proved useful in maintaining viability (up to 3 months) and promoting regeneration of non-encapsulated PLBs of *Darwinara*. The present findings that 'seeds' prepared using green PLBs germinated with 100% frequency support those of Sakamoto *et al*. (1995), who proposed that attainment of phototrophic nature and/or storage of reserve materials, which support embryo development, are critical factors for a high frequency conversion.

A variety of natural or synthetic polymers are available for use as gel matrix of synthetic 'seeds' (Barbotin *et al*., 1993). Gellation with agar and gellan gum require a heating procedure which is not conducive for the survival of encapsulatable units. With synthetic polymers (cross-linked polyacrylamide), it (gellation) is associated with exothermic reactions and/or formation of radicals. which are highly toxic for encapsulatable units. Polysaccharides better results because they are formed by an ionotropic reaction, which requires moderate conditions for complexation than those required by other gelling agents (Sakamoto *et al*., 1995). Presently sodium salt of alginate (Na-alginate) was chosen because of moderate viscosity and low spinnability of the solution, low toxicity, and quick gellation (Onishi *et al*., 1994); it is easily available at low cost. Moreover, rigidity of the alginate capsule provided better protection to the encased PLBs.

The shape, size, and texture of the 'seeds' were markedly influenced by the concentration of Na-alginate and the complexation period. The 'seeds' were spherical, firm, and non-leaky with 4% Na-alginate, 75mM calcium chloride, and complexation period of 30 min. The 'seeds' formed with lower concentrations of Na-alginate were fragile, irregular, and leaky, whereas those formed with higher concentration of Na-alginate and calcium chloride were rather hard and beaked. Poorly formed 'seeds' at lower concentrations of Na-alginate have been attributed to the impaired gelling ability of the alginate due to exposure to high temperature during autoclaving (Larkin *et al*., 1988). Perusal of literature, however, shows a great variation in the concentration of alginate used for encapsulation. Appropriate concentration seems to vary with the species and the nature of the propagule: 1.5% for *Daucus carota*

somatic embryos (Sakamoto *et al*. 1995), 2% for alfalfa somatic embryos (Redenbaugh *et al*., 1987), 2.5% for *Spathoglottis plicata* protocorms (Singh, 1991), *Aerides multiflorum* PLBs (Vij *et al*., 1993), and *Phaius tankervilliae* PLBs (Malemnganba et al., 1996), 3% each for *Dendrobium* cultivars (Saiprasad and Polisetty, 2003; Siew et al., 2014) and *Aranda* hybrid (Gantait et al., 2012), 4% for *Spathoglottis plicata* PLBs (Nayak *et al*., 1998) and *Geodorum densiflorum* PLBs (Datta *et al*., 1999), and 5-6% for *Atropa belladona, Dioscorea floribunda, Hyoscyamus muticus, Mentha arvensis, Picrorhiza kurroa,* and *Valeriana wallichii* shoot-buds (Ahuja *et al*., 1989). The required concentration may also vary with the alginate quality, which differs from brand to brand (Nayak *et al*., 1998) and batch to batch (Ahuja *et al*., 1989). The effective concentration of calcium chloride likewise varies from 50 mM (Singh, 1991; Datta *et al*., 1999) to 75mM (Malemnganba *et al*., 1996; Nayak *et al*., 1998; Saiprasad and Polisetty, 2003; present studies) to 100 mM (Redenbaugh and Ruzin, 1989).

Incidentally, the conversion frequency of the 'seeds' was observed to vary with the passage of time, conditions of their storage, and the nature of the sowing substratum. The freshly formed 'seeds' germinated readily with 100% frequency, but showed a progressive loss in conversion frequency with increase in storage period; the loss was more pronounced in 'seeds' stored at 4°C. While the 'seeds' stored at 25°C could retained viability for 30-60 days, those stored at 4°C remained viable for 45-120 days, depending upon the species. Similarly, Saiprasad and Polisetty (2003) observed that viability of the 'seeds' stored at 4°C varied with the species; it was 30 days for *Cattleya*, 60 days for *Oncidium*, and 75 days for *Dendrobium.* The high viability percentage of the 'seeds' stored at 4°C may be attributed to the low metabolic activity at this temperature. The conversion frequency of synthetic 'seeds' was better on agar-gelled medium than other substrata such as liquid medium irrigated sand, moss, epiphytic compost etc. Interestingly, as the encapsulated PLBs invariably proliferated during germination, production of synthetic 'seeds' is a novel technology simulating inherent polyembryonate potential of orchid seeds.

Onishi *et al*. (1994) observed that encapsulated embryos could not emerge from the gel bead ('seed') even if the embryos had excellent quality, and attributed this inhibition of conversion to unsuitable elasticity and/or strength of the gel bead, and oxygen deficiency within the gel bead. To overcome this problem a novel self-breaking or self-splitting gel bead has been developed by Sakamoto *et al*. (1992). In this method, alginate beads obtained after complexation are rinsed thoroughly with tap water (to wash out excess Ca^{2+} ions) and then immersed in monovalent cation solution (e.g. potassium nitrate; K^{+} ions partially substitute for Ca^{2+}), followed by another rinsing with running tap water. After sowing in humid conditions, this gel bead gradually swells, becomes brittle, and finally split spontaneously.

CONCLUSIONS

The present study demonstrated that the germination percentage of encapsulated PLBs was affected by the concentration of the complexing gel and duration of exposure to calcium chloride solution. Using 4% sodium alginate solution and maintaining in 75 mM calcium chloride solution for 30 min gave the highest percentage of germination. The result so obtained from encapsulation of PLBs can be used as a potential method to solve the problems of propagation for monopodial orchids like *Renanthera imschootiana* that have tiny seeds and lack of endosperm. The synthetic 'seeds' can be used for short-term storage, transport, and transplantation of tissue culture raised propagules in orchids, but their utility in direct transplantation of propagules into the greenhouse or soil shall remain limited until following aspects are achieved: i) synchrony and vigour in conversion, ii) storage ability (shelf-life) as long as seed; and iii) tolerance against drying after sowing.

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