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Regeneration of Encapsulated Protocorm Like Bodies of Medicinally Important Vulnerable Orchid *Flickingeria nodosa* (Dalz.) Seidenf

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Abstract: *Flickingeria nodosa* (Dalz.) Seidenf is an medicinally important epiphytic orchid. In this study, various concentrations of sodium alginate solutions and calcium chloride solutions were tested in order to optimize the shape, texture and time required for complexing of alginate synthetic seeds or beads for *Flickingeria nodosa* through encapsulation of Protocorm Like Bodies (PLBs). The developed PLBs on Burgeff's N3F basal medium were used as a source material for encapsulation. The sodium alginate was prepared with Burgeff's N3F basal medium which was used as a matrix for complexing. It was observed that 2% sodium alginate dipped in 100 mM $CaCl_2$ solution and incubated for 30 min in orbital shaker was found to be the best matrix and complexing agent respectively to produce firm, transparent and uniform synthetic seeds. The synthetic seeds were stored at 4°C for 3 months to study their viability. Further the encapsulated PLB's when cultured on 1X Burgeff's N3F medium, 2% sucrose fortified with 2 mg L^{-1} adenine sulphate and IAA 1 mg L^{-1} showed 95% germination response. The complete plantlets were successfully established with 85% survival frequency after a few days of indoor acclimatization and hardening. This technology can be adopted for *ex-situ* germplasm conservation of medicinally important vulnerable orchids.

Key words: *Flickingeria nodosa* (Dalz.) Seidenf., PLBs, Sodium alginate, $CaCl_2$, Burgeff's N3F medium

INTRODUCTION

Flickingeria nodosa (Dalz.) Seidenf. is a medicinally important, epiphytic orchid found in Eastern Himalayas, Srilanka and in parts of Western Ghats in India, namely Kodagu, Hassan, Uttara Kannada and Udumbansholai (Abraham and Vatsala, 1981; Rao, 1998). It was formerly referred to as *Ephemerantha macraei* [Lindl] (Rao, 1998). It is fast disappearing from its natural habitat by over exploitation by humans and status is vulnerable (Kumar *et al*., 2001). Hence a fast method of growing and conserving it in the green house is an urgent need.

Orchids grow in nature through seeds but in absence of mycorrhizal association they don't germinate in adequate numbers, so it remains as a vulnerable species. These obstacles may be overcome by adopting tissue culture technique. For appropriate germination of orchid seeds, *in vitro* propagation is imperative for multiplication rather than *in vivo*.

Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions and that retain this potential also after storage (Ara *et al*., 2000). The synthetic seed technology has gained considerable attention in plant biotechnology as a potential cost effective clonal propagation system (Pattanaik and Chand, 2000; Saiprasad, 2008). The synthetic seeds of orchids are produced by encapsulation of PLBs in alginate matrix (Mohanraj *et al*., 2009).The coating protects the PLBs from mechanical damage during handling and allows germination and conversion to occur without inducing undesirable variations .

However, there are no reports available at present on the development of synthetic seed for *Flickingeria nodosa* (Dalz.) Seidenf. Hence, the present study was undertaken to standardize the protocol for encapsulation using various concentrations of sodium alginate and calcium chloride solutions in order to optimize the shape, texture and time required for complexing of alginate synthetic seeds or beads for *Flickingeria nodosa* through encapsulation of Protocorm Like Bodies (PLBs).

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MATERIALS AND METHODS

The present study was conducted during the period from 1 Dec., 2010 to 30th Sep., 2011 at Genohelix Biolabs-A division of CASB, Jain University, Chamarajpet, Bangalore, Karnataka, India. The work is a part of Ph.D research of the corresponding author from Karpagam University, Coimbatore, Tamil Nadu, India.

The chemical which are used in the present study are of "A.R grade" chemicals from Nice, sd fine , Qualigens, Hi-media, Spectrochem, Colloids and Loba chemie.

The mature pods of *Flickingeria nodosa* (Dalz.) Seidenf. were harvested from plant maintained at green house of Genohelix Biolabs, washed thoroughly under running tap water, surface sterilized using liquid detergent 2% (v/v) savlon, 6-8 drops of tween-20 for 15 min, rinsed with 70% ethanol for 30 sec, disinfected with 0.05% (w/v) $HgCl_2$ for 6 min and rinsed in sterile water several times to remove the traces of $HgCl_2$. The sterilized pods were longitudinally excised on a sterile petriplate and the seeds were scooped out and seeds were inoculated on Burgeff"s N3F basal medium (Withner, 1955) with 2% sucrose. The cultures were maintained in the culture room at a temperature of 25±2°C, light intensity of 1000 LUX, relative humidity between 50-60%, under photo-periodic regime for 16 h light and 8 h dark cycle. After 32 days of incubation spherule shape structure developed. After 45 days of culture the PLBs were used for encapsulation.

PLBs were mixed in sodium alginate 2-4% (w/v) based Burgeff"s N3F basal medium with 2% sucrose.(w/v) separately. For gel complexation, 25-100 mM $CaCl_2$ solutions were prepared separately using distilled water. Both gel matrix and complexing agents were autoclaved at 121°C for 15 min at 15 pounds after adjusting the pH to 5.8. The gel complexation was carried out by picking up the PLBs along with matrix and dropping these into complexing agent $CaCl_2$ solution separately and incubated on orbital shaker at 100 rpm for different time intervals 15-60 min, where in ion exchange process takes place. The beads were recovered by decanting the $CaCl_2$ solution and washed several times with sterile water to remove traces of calcium chloride. The uniform beads thus obtained were stored at 4°C in sterile water for a period of 3 months to study their viability.

The encapsulated PLBs were inoculated on 1X Burgeff"s N3F basal medium with 2% sucrose and 1% agar fortified with 2 mg L^{-1} adenine sulphate and 1 mg L^{-1} IAA. The cultures were incubated in the culture room under controlled conditions for regeneration.

RESULTS

The first response of asymbiotic seed germination was noted by the change in shape and colour of the seeds. The seeds were found swollen within 20 days of culture that attained spherule shape structures after 32 days of culture. These spherule shape structures soon turned green in colour and developed into Protocorm like Bodies (PLBs) after 45 days of culture.

It was observed that 2% sodium alginate dipped in 100 mM $CaCl_2$ solution and incubated for 30 min in orbital shaker was found to be the best matrix and complexing agent respectively to produce firm, transparent and uniform synthetic seeds when compared to the other concentration, combinations and time (Table 1, Fig. 1a).

Table 1: Effect of different concentration and combination of sodium alginate, calcium chloride and exposure time on encapsulation of PLBs

Sodium alginate (%)	Calcium Chloride (mM)	Time intervals (min)	No. of beads produced	Quality of beads
2	25	15	20	Very soft
		30	20	Very soft
		45	20	Very soft
		60	20	Very soft
	50	15	20	Very soft
		30	20	Very soft
		45	20	Very soft
		60	20	Very soft
	75	15	20	Soft and transparent
		30	20	Soft and transparent
		45	20	Soft and transparent
		60	20	Soft and transparent
	100	15	20	Soft and transparent
		30	20	Firm and transparent
		45	20	Firm
		60	20	Firm
4	25	15	20	Soft
		30	20	Soft
		45	20	Soft
		60	20	Soft
	50	15	20	Firm
		30	20	Firm
		45	20	Firm
		60	20	Firm
	75	15	20	Slightly hard
		30	20	Slightly hard
		45	20	Slightly hard
		60	20	Slightly hard
	100	15	20	Slightly hard
		30	20	Slightly hard
		45	20	Slightly hard
		60	20	Slightly hard
6	25	15	20	Hard
		30	20	Hard
		45	20	Hard
		60	20	Hard
	50	15	20	Very hard
		30	20	Very hard
		45	20	Very hard
		60	20	Very hard
	75	15	20	Very hard
		30	20	Very hard
		45	20	Very hard
		60	20	Very hard
	100	15	20	Very hard
		30	20	Very hard
		45	20	Very hard
		60	20	Very hard

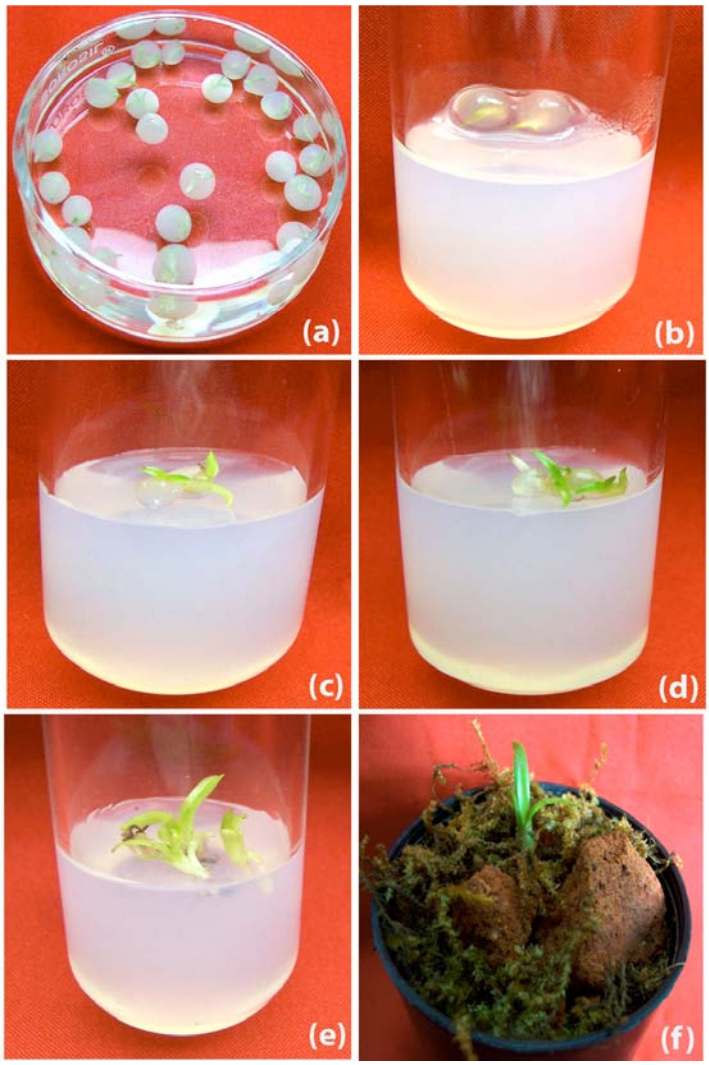

Fig. 1 (a-f): (a) Encapsulated PLBs with 2% sodium alginate dipped in 100 mM $CaCl_2$ solution and incubated for 30 min, (b-e) Different stages of germination of encapsulated PLBs on 1X Burgeff's N3F medium, 2% sucrose fortified with 2 mg L^{-1} adenine sulphate and NAA 1 mg L^{-1} and (f) Hardened plantlet

The synthetic seeds stored at 4°C remained viable and germinated up to 90 days. However, poor germination of 8% was observed after completion of 90 days of storage. Gradual declination of germination of synthetic seeds was observed when storage time was increased. After 0, 15, 30, 45, 60, 75, 90 days storage at 4°C, the conversion frequencies were 95, 83, 68, 49, 31, 19 and 6.5%, respectively.

The germination of the encapsulated PLBs were observed on 1X Burgeff"s N3F basal medium with 2% sucrose and 1% agar with growth regulators 2 mg L^{-1} adenine sulphate and 1 mg L^{-1} IAA after 10 days of culture and it showed 95% of germination. The shoot development was observed after 20 days of culturing and the well developed plantlets were seen after 60 days of culture (Fig. 1b-e). The plantlets were placed on pots containing brick, charcoal and moss and were maintained in the hardening chamber under controlled conditions. The hardened and acclimatized complete plantlets (Fig. 1f) were successfully established in the green house with 85% survival frequency.

DISCUSSION

The attempt to encapsulate orchid protocorms has been done on several plant species to produce synthetic

or artificial seeds (Sarmah *et al*., 2010; Mohanraj *et al*., 2009; Sharma *et al*., 1992). In the present investigation, it was noticed that the encapsulation of the PLBs with varied concentration and combination of sodium alginate and calcium chloride with time varied considerably . A 2% and 4% solution of sodium alginate upon complexation with 100 mM calcium chloride produced clear transparent firm capsules with an ion exchange duration of 30 min (Table 1, Fig. 1a). But when compared to 4% of sodium alginate 2% sodium alginate showed better result of regeneration which does not agrees with the findings of Mohanraj *et al.* (2009). A similar result have been observed in the encapsulation of PLBs of *Pecteilis gigantea* (Usha, 1997). The concentration above 4% of sodium alginate with all the concentrations of calcium chloride was very hard for the PLBs to regenerate this agrees with the findings of Mohanraj *et al.* (2009) and Saiprasad and Polisetty (2003). Lower concentration of the calcium chloride did not support the proper ion exchange for firm bead formation which agrees with the findings of Sarmah *et al.* (2010). The synthetic seeds stored at 4°C for an longer duration have reduced the viability of the encapsulated PLBs, this agrees with the findings of Sarmah *et al.* (2010). It is thought that the decline in the germination percentage observed among encapsulating propagules stored for a period of 3 months may be due to inhibited respiration of plant tissues by alginate leading to loss of viability (Bajaj, 1995).

CONCLUSION

Synthetic seed production and regeneration can be one of the promising approaches for conservation of vulnerable orchids. In the present study a reproducible protocol was developed for encapsulation and regeneration of vulnerable orchid *Flickingeria nodosa*.

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