ISSN 1996-0700

Asian Journal of Biotechnology

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Asian Journal of Biotechnology 1 (3): 124-128, 2009
ISSN 1996-0700

Production and Storage of Synthetic Seeds in *Coelogyne breviscapa* Lindl

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Abstract: The present investigation was carried out for the production of synthetic seeds from *Coelogyne breviscapa* Lindl. through encapsulation of Protocorm Like Bodies (PLBs). The effect of storage on the regeneration of the synthetic seeds was also studied. Sixty days old PLBs established from embryo cultures were encapsulated in 3% sodium alginate matrix and stored for 30 and 60 days and were germinated on three different substrates. Emergence of the leaf was observed within 12 days in Murashige and Skoog (MS) medium containing growth regulators. Among the growth regulators used, 2 mg L^{-1} of Indole Acetic Acid (IAA) was found to be the best for seedling growth. The encapsulated PLBs when stored at 4°C for 60 days showed no reduction in viability. Non encapsulated PLBs showed no viability at 4°C when stored for 15 days. Also, the germination percentage of artificial seeds stored at RT was always much lower in comparison to those stored at 4°C. This indicated the efficiency of low temperature for storage of artificial seeds. The germination percentage of encapsulated PLBs decreased gradually with increase in storage time.

Key words: Orchids, artificial seeds, encapsulation, PLBs

INTRODUCTION

Orchids constitute an order of royalty in the world of ornamental plants and they are of immense horticultural importance. They occupy the top position among the flowering plants, in cut-flower production and as potted plants, which fetch a very high price in the international market.

In the natural environment the seedling orchids are found only rarely, usually at the bases of mature orchid plants. The difficulties for germination of the seeds in their natural habitat can be best appreciated after an examination of an orchid pod which contains thousands of powdery seeds that rarely germinate. The seeds lack a metabolic machinery and functional endosperm and require a fungal association for germination.

Currently, the main method used by orchid breeders to germinate orchid seeds is the asymbiotic method. In this method, seeds are cultured aseptically on a nutrient medium supplemented with s simple carbon source like sucrose (Tan *et al*., 1998). This asymbiotic germination of orchid seeds has been successful for many species. During seed germination the embryo first forms tuberose protocorm PLBs, from which the complete plant develops.

Development of artificial seed production technology is currently considered as an effective and alternate method of propagation in several commercialy important agronomic and horticultural crops. It has been suggested as a powerful tool for mass propagation of elite plant species with high commercial value (Saiprasad, 2008). It is the most effective technique for the propagation of plant

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species that have problems in seed propagation and plants that produce non-viable seeds (Daud *et al*., 2008). Currently, systems of artificial seed production have progressed substantially, the most advanced being in seeding under *ex vitro* or field conditions, obtaining high percentages of conversion to plants (Nieves *et al*., 2003).

As the orchid industry is reliant on micropropagation as a major source of planting material, orchid synthetic seeds are indispensable as they could be delivered easily like true seeds from commercial tissue culture laboratories to growers, Hew and Young (1997). Synthetic seeds of orchids are produced by encapsulation of Protocorm Like Bodies (PLBs) in an alginate matrix. This system serves as a low-cost, high-volume propagation system (Saiprasad and Raghuveer, 2003). Developing a synthetic seed system by encapsulating protocorms for orchids can obviate the routine high cost propagation. Since, encapsulated PLBs can be directly sown in soil bypassing *in vitro* steps, synthetic seed system can revolutionize propagation and transportation of orchid germplasm (Singh, 2006).

If encapsulated PLBs can be stored for a long duration and at different temperatures, it will greatly enhance the efficiency of micropropagation by this system. Storing suggests a new means of cryopreservation (Surenciski *et al*., 2007). Uptil now, synthetic seed production by encapsulating PLBs has been achieved in only a few orchids. Keeping the above background in mind, the present study was carried out with the objective of producing artificial seeds in *Coelogyne breviscapa*, through PLBs and studying the effect of storage on encapsulated PLBs as a measure of *in vitro* conservation.

MATERIALS AND METHODS

The study was carried out at the Department kf Botany, Tissue Culture Lab., Bharathiar University, Coimbatore during 2001-2002. For production of PLBs, mature undehisced capsules of *C. breviscapa* were collected from Yerkaud, Tamil Nadu and were washed thoroughly with Teepol (BDH, India) under running tap water. They were then surface-sterilized with 3% sodium hypochlorite (Hi-Media, India) solution (v/v) for 15 min and were subsequently rinsed in sterilized double-distilled water. The capsules were cut longitudinally with the help of a sharp sterilized surgical blade and the seeds were inoculated in MS medium (Murashige and Skoog, 1962) supplemented with different growth hormones. The pH of the medium was adjusted to 5.6-5.8. The cultures were maintained at 25±2°C under 16 h photoperiod from cool-white-light giving 1000 lux at culture level. After 35 days following inoculation, green pin-head-like PLBs appeared.

For encapsulation of PLBs, 60 days following seed germination the PLBs were collected and washed in liquid MS medium. Sodium alginate (Hi-Media, India) solution (3%; w/v) was prepared by mixing with liquid MS medium. PLBs were mixed with sodium alginate solution and were subsequently singly dropped into an autoclaved-50mM solution of calcium chloride ($CaCl_{2\times}\ 2H_2O$) (Hi-Media, India). Calcium alginate beads were formed within 15-20 min on a rotary shaker moving at 80 rpm. Beads were taken out by decanting off the $CaCl_2$ solution, washed with sterilized double-distilled water and surface-dried with sterilized blotting paper. Freshly prepared beads were directly inoculated in MS medium and the concentrations of growth regulators being the same as the one which gave the best results when used during seed germination. The cultures were kept at same conditions as before.

A set of 150 artificial seeds was stored in dark at 4°C in sterile petri dishes, sealed with parafilm. They were taken out at regular intervals of 30 days and inoculated to see their germination percentage. Another set of 150 PLBs was kept at room temperature (25±2°C). Nonencapsulated PLBs were kept both at room temperature and at 4°C. Each treatment had 10 replicates and was repeated at least thrice.

RESULTS

The seeds of *C. breviscapa* were asymbiotically germinated on MS medium supplemented with various growth hormones. MS medium supplemented with 2 mg L^{-1} IAA was found to be the best suited for the formation of PLBs (Table 1). The greening of seeds commenced after 4 weeks and the protocorms differentiated within 35 days.

Freshly encapsulated PLBs (without storage; control) when directly inoculated on MS medium supplemented with IAA (2 mg L^{-1}), showed induction of growth after second week. Subsequently, they emerged out by rupturing the alginate matrix and established contact with the media (Fig. 1, 2).

The encapsulated PLBs retained their viability even after storage for 90 days. However, the germination percentage of the encapsulated PLBs decreased gradually with increase in storage time. The germination percentage of artificial seeds stored at room temperature was always much lower in comparison to those stored at 4°C (Table 2).

Table 1: Effect of various growth hormones on the germination of the seeds of *C. breviscapa*

Medium composition	Time taken for the formation of PLBs (Days±SE)
MS+ 0.5 mg L^{-1} 1AA	40±2.4
MS+ 1 mg L^{-1} 1AA	37±1.6
MS+ 2 mg L^{-1} 1AA	35±1.3
MS+ 0.5 mg L^{-1} 1BA	61±2.3
MS+ 1 mg L^{-1} 1BA	58±2.1
MS+ 2 mg L^{-1} 1BA	54±2.0
MS+ 0.5 mg L^{-1} 24-D	55±1.7
MS+ 1 mg L^{-1} 24-D	51±2.2
MS+ 2 mg L^{-1} 24-D	48±1.8

Fig. 1: Germination of artificial seeds in MS medium

Fig. 2: Emergence of shoot system from artificial seeds

Table 2: Germination percentage of the encapsulated PLBs of *C. breviscapa* at different temperatures in different storage periods

Type of the artificial seed	Storage temperature (°C)	Storage time (days)	Germination percentage±SE
Encapsulated	No storage	No storage	95.0±1.04
Encapsulated	4	30	91.0±0.83
Encapsulated	RT	30	82.5±0.38
Encapsulated	4	60	88.0±0.75
Encapsulated	RT	60	66.4±1.35
Encapsulated	4	90	44.0±0.56
Encapsulated	RT	90	33.6±1.05
Non-encapsulated	4	30	00.0
Non-encapsulated	RT	30	00.0

DISCUSSION

Encapsulation of somatic embryos or shoot buds and subsequent retrieval of complete plantlets has ben reported in a number of species (Ghosh and Sen, 1994). However, there are only a few reports on the propagation of orchids using synthetic seeds. In the present investigation an attempt has been made to produce synthetic seeds by encapsulating protocorms and to study the effect of storage on the regeneration of protocorms in *C. breviscapa.*

In the present study it was found that MS medium supplemented with 2 mg L^{-1} IAA was the best for seed germination. Similar effect of the growth regulators was observed in *C. mossiae* (Ananthan, 2003). The same medium composition was also found to be the best suited for the germination of encapsulated protocorms. On the contrary in cultivars of pear (*Pyrus communis*) and Rocha, an increase in Benzyl Amino Purine (BAP) concentration resulted in a higher number of shoots per explant (Freire *et al*., 2002). The maximum frequency (91%) of conversion of encapsulated beads into plantlets was achieved on Murashige and Skoog (MS) medium containing 2.5 µM 6- benzyladenine (BA) and 0.5 µM á-Naphthalene Acetic Acid (NAA) after 6 weeks of culture (Faisal and Anis, 2007). The best morphogenetic response of the plantlets was when the encapsulated buds of *Aechmea fasciata* was cultured on MS medium supplemented with, 2 mg L^{-1} BA or 1.0 mg L^{-1} KN after eight weeks of culture (Badr-Elden *et al*., 2005). The reason for the above mentioned discrepancy could be because of the fact that the use of auxins like IAA in the present study for seed germination conditions increased germination percentage and coleoptile elongation (Rakoslavskayal *et al*., 1999). The effect of various auxins on seed germination process include direct and indirect evidences indicating the involvement of auxins in seed germination (Chiwocha *et al*., 2005).

In the present study it was observed that when lower concentrations of sodium alginate (1-2%) was used for encapsulation, the beads were too difficult to handle and at higher concentrations (above 4%), the beads were hard and this might hinder with the emerging shoot. Therefore, 3% was found to be the most effective for encapsulation in *C. breviscapa.* Similar results were reported by Saiprasad and Raghuveer (2003) for the encapsulation of PLBs in *Dendrobium*, *Oncidium* and *Cattleya.*

A noticeable feature of the encapsulated protocorms was their ability to retain the viability in terms of germination potential even after a considerable period of storage. Similar reports have been made in *Spathoglottis plicata.* Artificial seeds stored at 4°C in comparison with the ones stored at room temperature showed a higher germination percentage. This indicated the efficiency of low temperature for the storage of artificial seeds. The retention of high viability percentage up to 60 days may be due to the availability of nutrients within the gel matrix. The encapsulation matrix serves as a reservoir and supplies the essential nutrients to encased explants.

However, it was also noticed that the germination percentage of the encapsulated PLBs decreased gradually with increase in storage time. A decline in germination percentage with increased storage is probably due to an anaerobic environment in the capsule. This could be because of the fact that embryos are not developmentally arrested and they continue active respiration (De, 2007).

Protocol development for artificial seed production and storage of the same in *C. breviscapa* may be an useful addition to the storage and transplantation of precious and costly hybrid orchids as well as for conservation of endangered germplasms. The judicious and intelligent coupling of artificial seed technology with that of microcomputer in achieving automated encapsulation and regeneration of plantlets would tremendously increase the efficiency of encapsulation and production of homogenous and high quality artificial seeds and will thus revolutionize the current concept of commercial micropropagation method.

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