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Original Research Paper

# *In Vitro* Propagation of Endangered Orchid Taxa Using Alginate-Encapsulated Protocorm Like Bodies (PLBs)

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## ABSTRACT

In the present investigation various concentrations of sodium alginate and calcium chloride solutions were tested in order to optimize the size, shape and texture of alginate synthetic seeds or beads for *Rhynchostylis retusa,* an epiphytic medicinal orchid. The seven weeks old protocorm like bodies (PLBs) were encapsulated with 2 to 3% sodium alginate (w/v) in the BM medium and exposed to 50 to 100 mM calcium chloride solution ($CaCl_2.2H_2O$). It was observed that 3% sodium alginate dipped in 100mM $CaCl_2.2H_2O$ solution was incubated for 40 min in orbital shaker to produce firm, transparent and uniform beads. The synthetic seeds were stored at 4°C and 25°C for 6 months to study their viability on the plantlets regeneration ability. The germination percentage of encapsulated PLBs decreased gradually with increase in storage time. This technology can be adopted for ex-situ germplasm conservation of medically important endangered orchids.

## INTRODUCTION

The orchids represent a diverse group of geologically young plants, still in an evolutionary flux; they have outsmarted and outnumbered their counterparts by evolving higher level of specialization in their vegetative and reproductive traits and occupy the top position among the flowering plants in cut flower production and fetch a very high price in the International market. Despite the fact that the orchids produce large number of seeds and are bestowed with an inherent potential of vegetative reproduction, they do not form dominant vegetation anywhere in the world. Orchids are progressively losing their natural habitat and getting rarer with every passage of time due to poor regeneration and extensive commercial collection. They are heading towards extinction and figure prominently in the red data book prepared by International Union for Conservation of Nature and Natural Resources (IUCN 1991).

Morel (1960) demonstrated the possibilities of using apical meristem for micropropagating a variety of orchids. It is particularly useful in outbreeders like orchids which generate a great deal of heterozygosity in the progenies. The technique is, however, detrimental to the growth and development of mother plant as it requires the sacrifice of the entire new growth or the only growing point. It is, thus, desirable that an alternate and equally effective multiplication system should exist by activating adventitious meristems in organs, whose excision does not endanger the survival of the source plant.

Murashige (1978) suggested the possibility of encapsulating in a nutritive gel and using these 'synthetic seeds' for propagation purposes without endangering the soured plant. Synthetic seeds are the living-seed like structures derived from somatic embryos in *in vitro* culture after encapsulation by a hydrogel. The preserved embryoids are called synthetic seeds. Plant propagation using synthetic seeds developed from somatic embryos opens up new vistas in agriculture. Synthetic seeds make a promising technique for propagation of transgenic plants, non-seed producing plants, and polyploids with elite traits (Daud et al. 2008). Being clonal in nature the technique cut short laborious selection procedure of the conventional recombination breeding and the most advanced being in seedling and high percentage conversion to plants in field conditions (Nieves et al. 2003, Saiprasad 2008). Uptil now synthetic seed production by encapsulation has been achieved in only few orchids. Keeping this in view, the present report deals with preparation of synthetic seeds of *Rhynchostylis retusa* Bl. (Orchidaceae), a genus of fox tail orchid, and an important stem herb. The stem extract of *R. retusa* commonly known as 'Rasna' is used as expectorant for curing rheumatic diseases (Lawler 1984). Besides being victim of its own beauty and utility, *R. retusa* is progressively losing its natural habitat and heading towards extinction particularly in Sri Lanka (Wicramasinghe 1992).

## MATERIAL AND METHODS

*R. retusa* Lindl. plants were collected in nature from Garhwal Himalayas eastwards to Arunachal Pradesh (1000-1800m),

and grown under greenhouse conditions at Panjab University, Chandigarh. The foliar explants, harvested from stock plants, were sequentially surface sterilized with solutions of Streptomycin (0.1%, 20min), sodium hypochlorite (4%, 15min) and dip in ethanol (70%, 3sec) before rinsing with sterilized distilled water. These were segmented into two halves and inoculated on sucrose (2%) supplemented and agar (0.9%) gelled basal medium (Mitra et al. 1976) and its various combinations with 1-10 mg/L NAA (α-naphthalene acetic acid), BAP (6-benzyl amino purine) and KN (kinetin) for the production of protocorm like bodies (PLBs).

For encapsulation purposes 2, 2.5, 3 and 4 % sodium alginate (w/v) in the BM medium was tested. Organogenetic PLBs were selected of uniform size of 5mm and dispersed in the alginate solutions for 10 minute. The mixture was pipette dropped using a Pasteur pipette with tip cut off individually into the different concentrations of calcium chloride ($CaCl_2.2H_2O$ : 50, 75, 100mM) solutions, which was constantly agitated with teflon coated magnetic stirrer (Table 1).

When sodium alginate drops come in contact with calcium chloride solution, surface complexion begins and firm round beads are formed; each bead contains one PLB. The blobs were allowed to complex in solution for time period ranging between 10 and 40 min (Table 2). It was observed that the PLBs encapsulated with 2-2.5% alginate and exposed to 50-100mM $CaCl_2.2H_2O$ solution produced fragile and tailed beads. The beads were best formed (spherical and non-leaky beads) by using 3% sodium alginate and 100mM $CaCl_2.2H_2O$ with complexation period of 40 minutes (Table 2, Fig. 1) and blotted dry on filter paper. The beads were subsequently picked up with the help of a spatula dried on a filter paper.

After encapsulation, the artificial seeds were preserved at 4°C and 25°C for 15, 30, 45, 60, 120, 150 and 180 days respectively. Agar gelled nutrient (Mitra et al. 1976) medium and nutrient irrigated sterile sand were used as the sowing substratum. Some seeds were coated with autoclaved talcum powder to avoid leaking and stickiness. The problems of fungal and bacterial infections during greenhouse conversion of the seeds could be successfully overcome by the use of 1 mg/L Bavistin and streptomycin (0.1%) in the nutrient matrix but the anti-microbial caused impairement in the response frequency. Therefore, it is desirable to use BM irrigated sand. The liquid medium pH was adjusted to 5.6. The experiments were performed 2 times each having 8 replicates (i.e., a total of 16 per treatment). All the experimental manipulations were done under aseptic conditions and the cultures incubated at 25±2°C under 12 hr photoperiod of 3500 lux light intensity, were regularly observed.

**Study on cytology of regenerated plantlets:** The root from regenerated plantlets were harvested at 6.00 to 6.30 a.m. and fixed in farmers fluid (1: 3: 6; acetic acid: chloroform: alcohol) for 24 hrs and stored in 70% alcohol until use. The root tips were squashed in 2% aceto-carmine solution to find out the cytological status of the regenerants.

Table 1: Effect of different concentration of sodium alginate and dihydrate calcium chloride on beads formation.

| | Sodium alginate | | |
|---|---|---|---|
| $CaCl_2.2H_2O$ (mM) | 2.0% | 2.5% | 3% |
| 50 | + | + | ++ |
| 75 | + | +++ | +++ |
| 100 | + | +++ | +++ |

*observation taken after a 40 min complexation period; + refers to bead quality in terms of shape, size and firmness.

Table 2: Effect of treatment time for synthetic seeds formation.

| Medium | Treatment time (min) | Quality of 'seeds' | Remarks |
|---|---|---|---|
| 3% Na-Alginate + 100 mM $CaCl_2.2H_2O$ | 10 | + | Fragile&tailed seeds |
| | 20 | +++ | Fragile soft seeds |
| | 30 | +++ | Isodiametric soft |
| | 40 | ++++ | Isodiametric compact |

**Acclimatization of the plantlet**: After well-developed shoot and root formation, the plantlets (3cm tall) were transferred to semisolid medium containing only half strength macro and micro salts of BM (Mitra et al. (1976) medium; sucrose and vitamins were eliminated. The plantlets were kept in this condition until they were 4-5cm tall, and washed with lukewarm water before transferring to moss, pine bark, brick and charcoal pieces (1:1:1:1) mixture. Humidity was maintained by covering each pot with transparent polythene bag. Holes of increasing size were made in the bags to reduce the humidity level gradually. The bags were removed after 4 weeks and small plants in the pots were transferred from 90% shade to the sunlight. Spraying with fungicide (Bavistin 1%) twice a week was necessary to keep fungus off from the young plants. Survival rate of acclimatized plantlets was more than 90%. Fig. 6 shows an acclimatized plantlet.

## RESULTS AND DISCUSSION

The attempt to produce synthetic seeds was made on several plant species, however, there were only a few reports in orchids (Mohanraj et al. 2009, Sarmah et al. 2010). Explants such as shoot tips, axillary buds and somatic embryos are encapsulated in cryoprotectant material like hydrogel, sodium alginate, ethylene glycol and chitosan (Redenbaugh et al. 1991, Helal et al. 2011, Dhabhai &Anand 2012). Although, a variety of natural and synthetic polymers are avail-

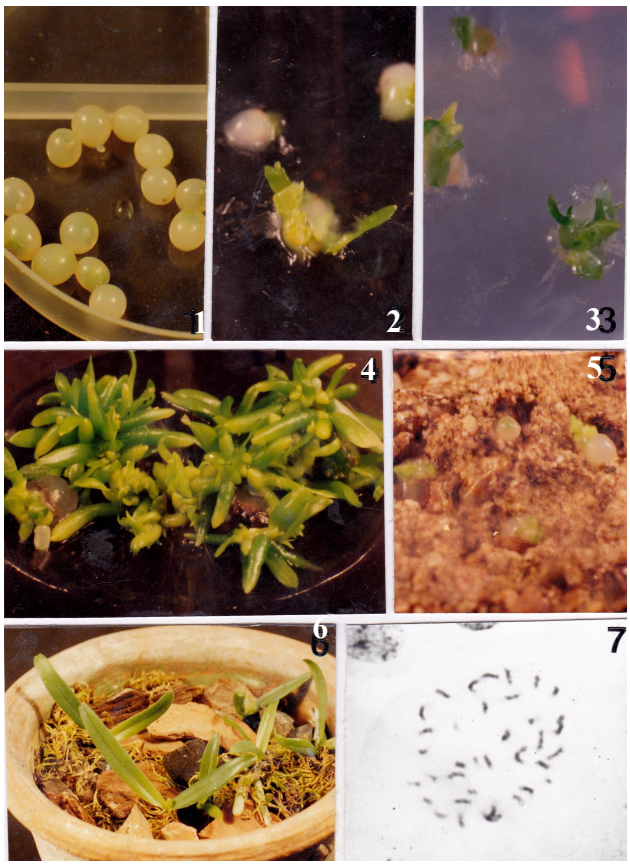


Fig. 1: Encapsulation PLBs with 3% Na-alginate in 100Mm $CaCl_2$ solution and complexed for 40 min; Figs. 2-3: Different stages of germination of encapsulated PLBs in BM medium; Fig. 4: Complete plantlets in BM + AC medium; Fig. 5: Beads in sterilized soi l; Fig. 6: Accilimatized plantlet; Fig.7: Cytological studies shows true to type plants (2n = 38).

able for encapsulation, sodium alginate is the most commonly used gel-matrix because of its easy gelling properties, non-toxicity, less viscosity and low cost (Redenbaugh et al. 1987, Mohanraj et al. 2009, Nagananda et al. 2011, Munni Devi et al. 2000). The coating protects explants during handling and allows conversion without inducing variations. In present investigation, an attempt has been made to produce synthetic seeds by encapsulating protocorm like bodies (PLBs) in *R. retusa* using different concentrations of sodium alginate and calcium chloride, which affect the shape and germination of artificial seeds. In the present study, a gelling matrix of 3% sodium alginate and 100 mM calcium chloride was found to be most suitable for formation of firm, clear and isodiametric ideal beads (Table 1). A similar result has been observed in the encapsulation of PLBs in *Flickingeria* (Nagananda et al. 2011), *Stevia* (Ali et al. 2012), Dendrobium 'Sonia' (Sai Prasad & Polisetty 2003). At lower concentrations of sodium alginate (2%) and calcium chloride (50-75 mM), beads were fragile and tailed beads, too soft to handle and failed to form isodiametric and defined shaped as it did not support the proper ion exchange while at higher concentrations of sodium alginate (4% to 5%) and calcium chloride (150 to 200 mM), beads were isodiametric but were hard enough to cause considerable delay in seed germination in compliance with earlier reports (Singh et al. 2006, Sarmah et al. 2010, Nagananda et al. 2011, Asmah et al. 2011). The conversion frequency of such 'seeds' was observed to vary with the passage of time and their storage i.e., inversely proportional to the period and temperature of storage and it was markedly influenced by the nature of sowing substratum. The synthetic seeds stored at 4°C in comparison with ones stored at room temperature (25°C) were more viable and showed a higher germination percentage. A reduction in 'synthetic seeds' due probably to the low metabolic rate at low temperature is in harmony with earlier studies (Bapat & Rao 1990, Redenbaugh et al. 1991). Gradual declination of germination of synthetic seeds was observed when storage time was increased due to inhibited respiration of plant tissues by alginate leading to loss of viability (Bajaj 1995). After 0, 15, 30, 45, 60, 120,150, 180 days of storage at 4°C in agar gelled nutrient medium (BM), the frequencies to plantlet conversion were 100, 100, 89, 89, 75, 75, 63 and 30% respectively (Table 3). These calcium alginate beads were regenerated into complete plantlets (Figs. 2-4). Fig. 5 depicts the germination of synthetic seeds in the BM irrigated sterilized sand. In BM irrigated sand, the germination efficiency was considerably reduced with prolonged storage (Table 3) and reduced to zero after 3 months at 4°C temperature whereas those stored at room temperature has shorter shelf life (Table 3). It is due probably to low metabolic rate at cold storage conditions and 'seeds' remained in a visual quiescent state that is helpful in preservation of nutrient reservoir in the synthetic seeds. Contrarily, those stored at room temperature lack quiescence and deplete nutrient reservoir, which resulted into low germination percentage (Nieves et al. 2001, Ikhaq et al. 2010).

Use of talc, though pronounced shelf life of 'seeds', adversely affects the conversion frequency of synthetic seeds (Dave et al. 2004). The genetic stability of plantlets growing after storage in encapsulated beads was confirmed via cytological studies which show true to the type plants (Fig. 7 ).

## CONCLUSION

Synthetic seeds as analog to natural botanic seeds comprising of meristematic tissue, have been increasingly realized since they are easy to handle and ensure economy of space, medium and time during lab to land transfer, potential of long term storage without losing viability and facilitate transport of germplasm and planting directly to

Table 3: Effect of temperature and periods of storage and sowing substratum on the conversion frequency of artificial seeds in orchid.

| Substratum | Storage temperature | 0 | 15 | 30 | 45 | 60 | 120 | 150 | 180 |
|---|---|---|---|---|---|---|---|---|---|
| Agar-gelled nutrient | 4°C | 100 | 100 | 89 | 89 | 75 | 75 | 63 | 30 |
| Mitra et al. (1976) medium | 25°C | 100 | 100 | 80 | 50 | 30 | - | - | - |
| BM irrigated sand | 4°C | 100 | 75 | 50 | 30 | 20 | 10 | - | - |
| | 25°C | 100 | 63 | 50 | 30 | - | - | - | - |

field conditions. Besides this, their free fertility with related genera offers exciting possibilities to progenate floriculturally significant crops. Since the encapsulated propagules invariably proliferate during germination, the production of synthetic 'seeds' is a novel technology representing natural extension of the somatic polyembryonic process. Moreover, they provide a significant alternative to the perpetual maintenance of live materials for the preservation of germplasm and contributed to a further improvement of cryopreservation procedures.

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