**ALGINATE-ENCAPSULATION, SHORT-TERM STORAGE AND PLANT REGENERATION FROM PROTOCORM OF *CYMBIDIUM BICOLOR* LINDL. AN EPIPHYTIC ORCHID.**

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**Abstract**

Artificial seeds of *Cymbidium bicolor* were produced by alginate encapsulation of 60 days old seed derived protocorms for propagation and short term storage. Clear isodiametric beads were obtained in 3% sodium alginate with 100 mM calcium chloride solution. Encapsulated protocorms exhibited the best re-growth and conversion frequency on MS medium supplemented with BAP (4.42μM). Encapsulated protocorms stored at 25°C were green and retained the viability with potential for conversion (52%) and germination even after 360 days. The encapsulated protocorms stored at 4°C remained viable up to 30 days beyond which the conversion rate decreased drastically. Well developed plantlets were transplanted into plastic pots containing vermiculite and maintained for 60 days in the culture room for acclimatization. 90% of the recovered plantlets were hardened off and established successfully in the soil. The present study could be useful for large scale propagation as well as short term storage of this commercial orchid.

**Keywords**: Epiphytic orchid, Synthetic seeds, Encapsulation, Protocorms and Germplasm preservation. *Cymbidium bicolor*

**Running title**: Alginate-encapsulation and short-term storage of *Cymbidium bicolor* lindl.

**INTRODUCTION**

Synthetic seed technology is currently considered as an effective and efficient alternate method of propagation in several commercial important agronomic and horticultural crops (Saiprasad 2001). It is also highly promising for the conservation and mass clonal propagation (Singh et al. 2006) of rare hybrids, elite genotypes, sterile unstable genotypes and genetically engineered plants for which seeds are either not available or that require a mycorrhizal-fungal association for their germination as in the case of orchids. Recently, encapsulation technology has attracted the interest of researchers for germplasm delivery and for various analytical studies (Ara et al. 2000). Synthetic seeds are prepared using unipolar structures such as hairy roots (Uozumi et al. 1992; Nakashimada et al. 1995), apical shoot tips (Rai et al. 2008; Singh et al. 2009), axillary buds (Ahmad and Anis 2010; Singh et al. 2010) and protocorm-like bodies of orchids (Sarmah et al. 2010), besides somatic embryos. Orchids are group of economically important plants valued for cut flowers. Synthetic seed technology has been employed for the mass multiplication and for the storage of number of the orchids ( Saiprasad et al. 2003; Nhut et al. 2005; Sarmah et al*.* 2010). The exploitation of the encapsulation technology has proven to be successful specifically for a number of Orchidaceae species, such as *Dendrobium wardianum* (Sharma et al. 1992), *Cymbidium gianteum* (Corrie and Tandon 1993), *Geodorum densiflorum* (Datta et al. 1999), *Spathoglottis plicata* (Khor et al. 1998), *Dendrobium densiflorum* (Vij et al. 2001), *Dendrobium*, *Oncidium*, and *Cattleya* (Saiprasad and Polisetty 2003), *Ipsea malabarica* (Martin 2003), *Vanilla planifolia* (Divakaran et al. 2006), *Vanda coerulea* (Sarmah et al. 2010), *Coelogyne breviscapa* (Mohanraj et al. 2009 ), *Aranda*×*Vanda* (Gantait et al. 2012), *Cymbidium devonianum* (Das et al.2011). *Flickingeria nodosa* (Nagananda et al. 2011), *Dendrobium Candidum (*Zhang and Yan2011), *Phalaenopsis bellina* (Khoddamzadeh et al. 2011), *Dendrobium nobile* (Mohanty et al. 2012) and *Dendrobium* Shavin White (Bustam et al. 2012).

*Cymbidium* or ‘‘boat orchid’’ is a popular orchid grown commercially worldwide (Chugh et al. 2009). Today, orchids such as *Cymbidium, Dendrobium, Oncidium* and *Phalaenopsis* are marketed globally and the orchid industry contributes substantially to the economy of many the South East Asian countries. *Cymbidium bicolor* Lindl. is an important horticultural orchid known for its beautiful flowers (Chugh et al. 2009). In *Cymbidium*, plantlets were regenerated in *in vitro* using shoot tips (Morel 1964), mature and immature seeds (Chung et al. 1985; Shimasaki and Uemoto 1990), green capsules (Hossain et al. 2010;Deb and Pongener 2011), flower stalks (Wang 1988), pseudo bulbs (Shimasaki and Uemoto 1990), shoot segments (Nayak et al. 1997), flower buds (Shimasaki and Uemoto 1990), protocorm-like bodies (PLBs) (Begum et al. 1994a; Huan and Tanaka 2004; Teixeira da Silva et al. 2007), thin cell layers of PLBs (Malabadi et al. 2008), artificial seeds (Nhut et al. 2005) and through somatic embryogenesis (Begum et al. 1994b; Chang and Chang1998; Huan and Tanaka 2004; Mahendran and Narmatha Bai 2012). Hoque et al. (1994) reported that Phytomax medium was the best among the five different media (Phytomax, Modified Vacin and Went, KC, KCM and LO medium) tested for large scale multiplication of *C. bicolor*. Mahendran et al. (2013) reported asymbiotic seed germination of *C. bicolor* and the influence of mycorrhizal fungus on seedling development. The present study was carried out with the aim to optimize the methods for the production of synseeds using seed derived protocorm for propagation and *in vitro* short-term storage. Broadly, three experiments were conducted (i) to assess the effect of encapsulation matrix on the formation of synthetic seeds (ii) to test the efficiency of growth regulators for *in vitro* conversion of synthetic seeds under aseptic conditions and (iii) to study the effect of storage (4°C and 25°C) temperature on the conversion of encapsulated protocorms.

**MATERIALS AND METHODS**

**Preparation of explants, surface sterilization and culture conditions**

Green pods of *Cymbidium bicolor* were collected from National Yercaud Orchidarium Salem, Tamilnadu. The freshly collected pods were surface sterilized in 0.001% mercuric chloride solution for 2 minutes, rinsed thoroughly thrice with sterile distilled water, dipped in 70% ethanol for 30 seconds and flamed. The surface sterilized capsules were cut open longitudinally with a sterile scalpel and the seeds were scooped out and inoculated on B5 medium in (22 × 120mm ) glass test tubes, containing 20 ml of the medium. Seeds were germinated in the culture room at 25 ± 2ºC with 80% relative humidity under white fluorescent tubes at an intensity of 50 µmol m-2 s-1 with 16/8-h L/D photoperiod. 60 days old protocorms developed on the above medium were used as explants for synthetic seed production.

**Preparation of beads**

For encapsulation, sodium alginate was prepared in the range of 1.0 2.0, 3.0, 4.0, or 5.0% (w/v), and calcium chloride (CaCl2.2H2O) solution in the range of 25, 50,75, 100 ,150 or 200 mM (w/v) in distilled water. The protocorms were mixed with alginate solution with the help of a sterile dropper and then gently dropped into 150 ml CaCl2.2H2O solution. The droplets containing a single protocorm was held for 20–30 min in the CaCl2.2H2O. After hardening, the alginate beads (6–7 mm) were taken out and washed 3–4 times with sterile distilled water. Freshly prepared beads were inoculated in B5 medium supplemented with cytokinins like 6- benzyl aminoputrine (BAP-1.10, 2.21, 4.42, 8.84 or 13.26 µM), N6-furfurylaminopurine (Kn- 1.16, 2.32, 4. 64, 9.28 or 13.92 µM), N phenyl-N’-1,2, 3-thiadiazol-5-ylurea (TDZ-1.13, 2.26, 4.52, 9.24 or 13.76 µM) or (Zeatin -1.12, 2.24, 3.36, 4.58 or 6.90 µM) individually.

**Short-term storage of encapsulated protocorms**

The encapsulated protocorms were stored at two different temperatures 4°C and 25 ± 2°C. After encapsulation, beads were transferred to sterile petriplates and tightly sealed with aluminium foil to prevent desiccation. The petriplates was then kept at 4 ºC or 25 ± 2°C and the encapsulated protocorms were stored for 0, 30, 60, 90, 180 and 360 days. After each storage period, beads were removed from the petriplate at regular intervals and cultured in B5 medium containing 4.43 µM BA at 25°C under light (16-h photoperiod at 50 mol m-2 s-1). The percentage of germination, number of protocorms and roots number were calculated after 4 weeks.

**Hardening**

Well-developed plantlets were rinsed thoroughly with tap water to remove residual nutrients and agar from the plant body and transplanted to plastic pot containing vermiculite. The plastic pots were covered by polyethylene bags and maintained two months inside the culture room for acclimatization under cool white tubular fluorescent lights (40 W, 220V, Philips Electronics India Ltd.) at 50 µmol−lm−2 s−1 with a 16 h photoperiod at 25± 2 °C.

**Data collection and statistical analyses**

Each experiment was conducted in completely randomized design. Each treatment was repeated thrice with five replicates with 5 synseeds for each tube. Data were subjected to analysis of variance (ANOVA) and Duncan’s Multiple Range Test (DMRT) using SPSS 17.0 version used to test the significance of difference among the treatments.

**RESULTS AND DISCUSSION**

The principle involved in the alginate encapsulation process is the ion exchange between sodium (Na+) and calcium (Ca++) ions. The formation of beads and the subsequent success of the encapsulation depend on the concentration of alginate and calcium chloride used and it may vary with different propagules as well as with the different plant species (Sharma et al.2012). Hence, the concentrations of these two solutions and complexion time must be optimized for the formation of an ideal bead (Saiprasad 2001). In most of the reports, 3% (w/v) sodium alginate and100 mM calcium chloride for 20–30 min has proved to be the best combination for the formation of an ideal synseed (Tabassum et al. 2010; Ahmad and Anis 2010; Ozudogru et al. 2011; Alatar and Faisal 2012; Hung and Trueman, 2011, 2012a,b;Gantait et al. 2012). However, 3% sodium alginate upon complexion with 75 mM calcium chloride for 20–30 min was found to be optimum combination for proper hardening of beads orchids such as of *Dendrobium*, *Oncidium* and *Cattleya* orchids (Saiprasad and Polisetty 2003). In contrast, for the encapsulation of nodal segments of medicinal plant such as *Pogostemon cablin* (Swamy et al. 2009) and *Spilanthes acmella* (Sharma et al. 2009b) and the microshoots of *Zingiber officinale* (Sundararaj et al. 2010), 4% sodium alginate with 100 mM calcium chloride was optimum. This variation in sodium alginate concentration for synseed formation in different plant species might be due to the variation of the source from which the chemicals were purchased as suggested by Ghosh and Sen (1994) and Mandal et al. (2000). In the present study, alginate-beads containing seed derived protocorm (beads) showed different morphology (clearness, form, and consistency) based on the concentrations of sodium alginate and calcium chloride used. Ideal beads were obtained with 3 % sodium alginate in 100 mM CaCl2 solution (Fig.1C). At lower concentrations (1–2%), sodium alginate became unsuitable for encapsulation because of a reduction in its gelling ability following exposure to high temperature during autoclaving (LarKn et al.1998). On the contrary, high concentrations of sodium alginate (5–6%) beads were isodiametric but too hard, causing considerable delay in sprouting of shoots (Ahmad and Anis 2010; Sharma et al. 2009ab; Gantait and Sinniah 2012), which was also observed in our study (Fig.1A-D).

Among the four cytokinins such as BAP, Kn, Zeatin and TDZ tested, multiple shoot induction was frequent in BAP (4.42µM), followed by Kn (13.92µM). Among the different levels BAP tested, the maximum number of shoots was observed on the B5 medium containing 4.42 µM of BAP (30.25±0.11) (Fig.1F) (Table 1). The shoot buds first appeared as small white protuberances over the surface of the protocorm which eventually developed into multiple shoots within 45–60 days. The number of shoot buds increased with increasing concentration of BAP up to an optimal level of 4.42µM. Among the various concentrations of Kn and TDZ tested, maximum number of the multiple shoots were recorded in B5 medium supplemented with 13.92 µM Kn (23.89±0.54shoots/ encapsulated explant), 13.76 µM TDZ (6.41±0.50shoots/ encapsulated explant) and 6.90 µM Zeatin (14.56± 0.23 shoots/encapsulated explant).

Supplementation of PGRs to the germination medium has been found to eliminate the requirement of an additional *in vitro* root induction step prior to acclimatization (Sharma and Shahzad 2012). In the present study, one-step plantlet formation was achieved using encapsulated protocorm without any specific root induction medium. MS medium supplemented with BAP (4.43µM) significantly increased the number of protocorms and induced roots (Fig.1E) (Table 1). As observed in *Cassava* (Danso and Ford-Lloyd 2003) and *Zingiber officinale* (Sundararaj et al. 2010). In contrast, Gangopadhyay et al. (2005) devised a two-step method to achieve maximum bead conversion in *A. comosus* (pineapple). In the first step, shoots were retrieved from encapsulated beads and in the second step, these microshoots were rooted in liquid medium (supplemented with 0.01 mM IBA and 0.002 mM Kn) supported with Luffa-sponge.

In *C. bicolor* the encapsulated protocorms stored at 25°C gave promising results for germination and conversion. Table-2 presents the germination and conversion competency of synthetic seeds stored at 4°C and 25°C for 30, 60, 90, 180 and 360 days. Germination percent of the encapsulated protocorms decreased gradually with an increase in storage duration at 25°C with being significantly more (*P*˂0.05) compared to that stored in 4°C. Encapsulated protocorms stored at 4°C up to 30 days retained their viability (10.23%) and with the advancement of storage duration (60 days) the synthetic seed turned necrotic, shrunken and brown, resulting in complete death. Whereas, beads stored at 25°C were green, with potential for conversion and with 52% germination even at 360 days. Storage of encapsulated protocorm/PLBs is greatly influenced by the temperature. However, the response of synthetic seeds to storage temperature appears to be species specific (Bustam et al. 2012 and Gantait and Sinniah 2012). The failure of prolonged storage in 4°C in the present study corresponds to the earlier reports where, in low temperature (4°C) storage, the storage life of synthetic seed was rather short (Redenbaugh et al. 1987; Gantait et al. 2012). Similarly, the germination of encapsulated PLBs of *Aranda* x *Vanda coerulea* also showed marked decline, following storage at low temperature (Gantait et al. 2012). Storage at room temperature (25°C) implemented in this study was effective for short-term storage, and handling without refrigerated containers and beads stored up to 360 days gave considerable conversion rate (52 %). Similarly, in *Aranda* x *Vanda coerulea* beads stored at 4°C showed rapid deterioration and faced complete death within 160 days while those stored for 200 days at 25 °C showed relatively high conversion (71.6 %) ( Gantait and Sinniah 2012). Encapsulated PLBs of *Dendrobium* were successfully stored up to 135 days with 52% survival at 25±2 °C (Bustam et al. 2012). In *Vanda coerulea* encapsulated PLBs stored at 4°C retaining their viability up to 100 days (Sharmah et al. 2010) and in *Phalaenopsis bellina* beads stored at 25°C up to 60 days did not show any response (Khoddamzadeh et al.2011). The decline in the viability and germination rate of stored encapsulated protocorms (4 °C) may be related to oxygen deficiency in the gel bead as reported by earlier researchers (Danso and Ford-Lloyd 2003; Gantait et al. 2012).

The conversion efficiency and the number of seedling/ protocorms and shoots length declined with increase in storage duration at both 4 °C and 25 °C of plant markedly reduced with an increase in storage time. Till 30 days of storage at both 25 °C and 4°C, number of shoots reduced from 30.25±0.11to 19.12± 0.71while the shoot length reduced from 2.58±0.74 to 2.29±0.97.At 360 days of storage, capsules stored at 25°C showed 18.87±0.44 protocorm/ bead, whereas, capsules stored at 4°C lost their viability completely after 30 days.Similarly, In relation to morphological changes, the number of protocorms, root numbers, shoot and root length of the stored synthetic seed on optimized conversion medium decreased in a linear manner with increase in storage duration till 360 days. Root numbers on the other hand, was unaffected by the storage duration under refrigerated conditions (4°C), as each encapsulated protocorms produced one root after 30 days of storage time. While storage at 25°C had a different trend with an increase in storage time, a linear reduction in root number was observed (1.00 to 0.53) up to 180 days of storage (Table 2). According to Danso and Ford-Lloyd (2003) and Gantait et al. (2012) the decline in conversion or morphogenesis i.e. shoot forming capacity as the result of prolonged storage could be due to inhibited respiration of tissues.

The plantlets were transferred to the potting medium containing vermiculite and covered with polythene bag and maintained at 25±2°C. After 2 months, the cover was gradually loosened, thus dropping the humidity (65–70%) (Fig.1G) this procedure subsequently resulted in *in vitro* hardening of the plants. Acclimatization of plants grown in *in vitro* to natural conditions is a critical step for many species, requiring time and expensive installation that restrict the commercial application of micropropagation process. About 90 % survival rate in the present study was obtained when plantlets were transferred to field (Fig.1H).

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