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Encapsulation of Protocorm of *Cymbidium bicolor* Lindl. for Short-Term Storage and Germplasm Exchange

Ganesan Mahendran
Research scholar, Department of Botany, Bharathiar University, Coimbatore- 641046, India.

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*Corresponding author's email: mahendran0007@gmail.com

Abstract

The present study describes the encapsulation of protocorm of *Cymbidium bicolor* Lindl. from 60 days-old in seed cultures for short-term conservation and propagation. Various concentrations and combinations of gelling matrix (sodium alginate) and complexing agents (calcium chloride) were tested to prepare uniform beads. The ideal beads were obtained through a combination of 3% sodium alginate and 100 mM calcium chloride. Encapsulated protocorms exhibited the best re-growth and conversion frequency on MS medium supplemented with BA (4.42 µM). 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. The 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: Acclimatization, Encapsulation, Epiphytic orchid, Germplasm preservation, Protocorms, Sodium alginate, Synthetic seeds.

Abbreviations: ANOVA (Analysis of variance), SPSS (Statistical package for the social sciences), $CaCl_2.2H_2O$ (Calcium chloride), DDW (Double distilled water), TDZ (Thidiazuron), BA (6-Benzyl adenine), Z (Zeatin), Kn (6-Furfurylaminopurine).

INTRODUCTION

The encapsulation technique is an important application of micropropagation that offers the potential of easy handling, exchange of germplasm between laboratories, efficient short- or long-term storage and improves delivery of *in vitro* regenerated plantlets to the field or to the green house (Piccioni and Standardi, 1995; Chand and Singh, 2004; Rai *et al*., 2009). Synseed technology provides a means for the transportation of propagules to distant places as well as to different laboratories without a loss in vigor for shoot organogenesis in micropropagation programs (Rihan *et al*., 2011; Hung and Trueman, 2012a,b; Lata *et al*., 2012; Reddy *et al*., 2012). Therefore, appropriate storage conditions and a definite storage period are prerequisites to maintain synseed viability during transportation that leads to successful commercialization of synseed technology (Sharma and Shahzad, 2012; Sharma *et al*., 2013).

Synseedare 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). Orchids are a 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 and Polisetty, 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 Yan, 2011), *Phalaenopsis bellina* (Khoddamzadeh *et al*., 2011), *Dendrobium nobile* (Mohanty *et al*., 2012) and *Dendrobium Shavin White* (Bustam *et al*., 2013).

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*., 1994; 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 (Chang and Chang, 1998; 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*. 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

Explant source

Green pods of *Cymbidium bicolour* collected from National Yercaud Orchidarium Salem, India, were surface sterilized through the procedure adopted by Mahendran *et al*. (2013) and used

to raise aseptic protocorms in seed germination medium that comprised Lindemann orchid (LO) (Lindemann *et al*.,1970) under controlled conditions of light, temperature and humidity (Mahendran *et al.*, 2013). The protocorms were taken from *in vitro* seed cultures of *C. bicolor* and used as the source of explants for the preparation of synseeds.

Encapsulation matrix and complexing agent

Sodium alginate (Qualigens, India) was used as gelling agent and prepared in double distilled water (DDW) and liquid B5 medium (with 3% sucrose) at different concentrations, i.e., 1, 2, 3, 4 and 5% (w/v). For complexion, 25, 50, 75, 100 and 200 mM calcium chloride ($CaCl_2.2H_2O$) solution was prepared in liquid B5 medium. The pH of the gel matrix and the complexing agent was adjusted to 5.8 prior to autoclaving at 121°C for 20 min.

Encapsulation of explants

Encapsulation was accomplished by mixing the 60-day-oldprotocorms with sodium alginate solution and dropping them in $CaCl_2.2H_2O$ solution using a pipette. The droplets containing the explants were held at least for 25–30 min to achieve polymerization. The alginate beads containing the protocorms were retrieved from the solution and rinsed twice with sterilized DDW to remove the traces of $CaCl_2.2H_2O$ and transferred to sterile filter paper in Petri dishes for 5 min under the laminar airflow cabinet to eliminate the excess of water and thereafter transferred to culture vials containing nutrient medium.

Planting media and culture conditions

The encapsulated protocorms (alginate beads) were transferred to test tube (Borosil, India) containing B5 basal medium without plant growth regulator (PGR) and B5 medium supplemented with cytokinins like BA (1.10, 2.21, 4.42, 8.84 or 13.26 µM), Kn (1.16, 2.32, 4. 64, 9.28 or 13.92 µM), TDZ (1.13, 2.26, 4.52, 9.24 or 13.76 µM) or Zt (1.12, 2.24, 3.36, 4.58 or 6.90 µM), individually. The culture medium was gelled with 0.8 % (w/v) agar and pH was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. Cultures were maintained at 25 ± 2°C under 16/8 h light–dark conditions with a photosynthetic photon flux density (PPFD) of 50 µmol m-2 s-1 provided by cool white fluorescent tubes (40 W, Philips, India).

Short-term storage of encapsulatedprotocorm

Two sets of 150 encapsulated protocorms were kept in two sterile wide mouth culture flask (Borosil, India) properly covered with aluminium foil and stored in refrigerator at 4°C and culture room at 25 ± 2°C. Six different exposure times (0, 30, 60, 90, 180 and 360 days) were evaluated for conversion of synseeds into plantlets. After each storage period, 15 encapsulated were transferred to B5 medium containing optimal concentration of PGRs for conversion into plantlets. During storage period the beads were sprayed with sterile DDW after every storage period to ensure the moist conditions so that the beads may not shrink by losing water. The number of shoot regeneration, root, shoot and root length of encapsulated protocorm was recorded after 8 weeks of culture.

Hardening and acclimatization

Plantlets with well-developed root and shoot system were removed from the culture medium and washed gently under running tap water to remove any adherent gel from the roots and transferred to plastic cups containing sterile vermiculite. These were kept under similar culture conditions as mentioned earlier and covered with transparent polythene bags to ensure high humidity. These were irrigated after every 10 days with one-fourth strength B5 salt solution (without vitamins) for 2 month. Polythene bags were removed gradually after 1 month in order to acclimatize the plantlets and after 8 weeks they were transferred transplanted into plastic pot (10 x 8 cm; height x diameter) containing sterilized vermiculite (50 g per pot) and maintained in greenhouse under normal day light conditions.

Statistical analysis

The results were expressed as mean ± SE of three independent replicates of independent experiments. Data were subjected to analysis of variance (one way ANOVA) and Duncan's Multiple Range Test (DMRT) using SPSS version 17.0.

RESULTS AND DISCUSSION

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 and Shahzad, 2012). Hence, the concentrations of these two solutions and complexion time must be optimized for the formation of an ideal bead. 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), *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 $CaCl_2$ 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 (Larkin et al., 1998). On the contrary, high concentrations of sodium alginate (5–6%), beads were diametric but too hard, causing considerable delay in sprouting of shoots (Ahmad and Anis, 2010; Sharma *et al.*, 2009 a,b; Gantait and Sinniah, 2012) which was also observed in our study (Fig.1a-d).

The most desirable property of the encapsulated explants is their ability to retain viability in terms of regrowth and conversion abilities after encapsulation (Adriani *et al.*, 2000; Micheli *et al.,* 2007). In the present study, the ideal beads produced by encapsulating protocorm in 3% sodium alginate and 100 mM $CaCl_2.2H_2O$ were cultured on B5 basal medium without any PGR as well as with various concentrations of BA, Kn, Zt and TDZ (Fig.1e). Synseeds cultured on B5 basal medium exhibited 90% regeneration response and this occurred after 10 weeks of culture. Addition of higher concentration of cytokinins enhanced the regeneration potential of the beads and the shoots emerged out within 10-15 days of inoculation onto the regeneration medium. An average of 30.25±0.11 shoots/bead was produced in the medium containing 4.42 µM of BA with 87% regeneration response after 10 weeks of culture (Table 1; Fig. 1f). 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 BA up to an optimal level of 4.42 µM. 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±0.54 shoots/encapsulated explant), 13.76 µM TDZ (6.41±0.50 shoots/bead) and 6.90 µM zeatin (14.56±0.23 shoots/bead). Emergence of single or multiple shoots from the encapsulated explants has also been reported earlier in other medicinal plants (Mandal *et al.*, 2000; Lata *et al*., 2009; Shrivastava *et al.*, 2009). Mishra *et al*. (2011) also described that encapsulated explants of *Picrorhiza kurroa* exhibited simultaneous production of shoots and roots while rest of

the non-rooted shoots were transferred to root induction medium for the development of roots. Supplementation of PGRs to the regeneration 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, encapsulated explants of *C. bicolor* exhibited simultaneous production of shoots and roots without any specific root induction medium (Fig.1g). B5 medium supplemented with BA (4.43 µM) significantly increased the number of protocorms as well as induced roots (Table 1). Similar result 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.

Values represent mean±S.E. Each treatment was repeated twice and each treatment counted of 5 replicate culture tubes, each containing five encapsulated protocorms. Means in a column with the different letter (superscript) are significantly different according to DMRT ($p < 0.05$).

In *C. bicolor* the encapsulated protocorms stored at 25°C gave promising results for germination and regeneration. Table 2 presents the germination and conversion competency of synthetic seeds stored at 4°C and 25°C for 0, 30, 60, 90, 180 and 360 days. Regeneration percent of the encapsulated protocorms decreased gradually with an increase in storage duration at 25°C with being significantly more ($p \leq 0.05$) 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).Sim-

Table 1. Effect of different plant growth regulators on regeneration of encapsulated protocorm of *C. bicolor.*

BA (µM/l)	Kn (µM/l)	TDZ (µM/l)	Zt (µM/l)	Regeneration response (%)	No. of shoots/bead	Shoot length (cm)/bead	No. of Roots	Root length (cm)
-	-	-	-	90	2.43±0.12 ^{i}	3.21±0.23 ^{d}	1.87±0.89 ^{c}	2.87±0.43 ^{a}
1.10	-	-	-	92	10.23±0.34 ^{e}	2.11±0.14 ^{g}	2.12±0.43 ^{b}	2.56 ±0.11 ^{a}
2.21	-	-	-	97	16.65±0.98 ^{c}	2.54±0.62 ^{e}	2.11±0.98 ^{b}	2.11±0.65 ^{b}
4.42	-	-	-	87	30.25±0.11 ^{a}	2.58±0.74 ^{e}	1.11±0.43 ^{f}	1.15±0.13 ^{f}
8.84	-	-	-	98	20.17±0.13 ^{b}	2.33±0.54 ^{f}	1.06±0.11 ^{g}	1.00±0.55 ^{g}
13.26	-	-	-	79	17.12±0.34 ^{c}	3.32±0.12 ^{d}	0.80±0.21 ^{h}	0.59±0.63 ^{h}
-	1.16	-	-	89	3.56±0.78 ^{g}	2.32±0.65 ^{f}	2.61±0.12 ^{b}	1.29±0.63 ^{de}
-	2.32	-	-	90	7.87±0.91 ^{f}	2.12±0 .76 ^{g}	3.19±0.34 ^{a}	1.10±0.23 ^{f}
-	4.64	-	-	76	12.43±0.19 ^{d}	3.98±0.17 ^{b}	1.43±0.19 ^{e}	1.49±0.49 ^{d}
-	9.28	-	-	98	17.81±0.71 ^{c}	3.30±0.22 ^{d}	1.67±0.20 ^{d}	1.38±0.53 ^{d}
-	13.92	-	-	100	23.89±0.54 ^{b}	2.23±0.21 ^{f}	1.21±0.21 ^{f}	1.22±0.88 ^{e}
-	-	1.13	-	90	3.54±0.43 ^{h}	4.54±0.76 ^{a}	1.11±0.56 ^{f}	1.21±0.56 ^{e}
-	-	2.26	-	89	3.76±0.11 ^{g}	4.32±0.54 ^{a}	1.00±0.22 ^{g}	1.19±0.77 ^{ef}
-	-	4.52	-	100	4.12±0.61 ^{g}	3.80±0.27 ^{c}	1.12±0.31 ^{f}	1.87±0.60 ^{c}
-	-	9.24	-	90	5.45±0.99 ^{g}	3.76±0.76 ^{c}	0.95±0.93 ^{h}	1.69±0.93 ^{c}
-	-	13.76	-	90	6.41±0.50 ^{f}	2.34±0.41 ^{f}	0.81±0.21 ^{h}	1.11±0.09 ^{f}
-	-	-	1.12	87	2.45±0.81 ^{i}	3.50±0.16 ^{e}	2.52±0.75 ^{ab}	1.13±0.99 ^{f}
-	-	-	2.24	90	4.76±0.11 ^{g}	2.54±0.58 ^{e}	3.84±0.84 ^{a}	1.00±0.21 ^{g}
-	-	-	3.36	96	5.10±0.89 ^{g}	2.20±0.43 ^{f}	1.66±0.61 ^{d}	1.08±0.33 ^{g}
-	-	-	4.58	97	10.010.21 ^{e}	3.20±0.03 ^{d}	1.00±0.39 ^{g}	1.32±0.52 ^{d}
-	-	-	6.90	100	14.56± 0.23 ^{c}	3.60±0.36 ^{c}	1.41±0.14 ^{e}	1.12±0.79 ^{f}

Values represent mean±S.E. Each treatment was repeated twice and each treatment counted of 5 replicate culture tubes, each containing five encapsulated protocorms. Means in a column with the different letter (superscript) are significantly different according to DMRT ($p < 0.05$).

Table 2. Effect of storage at 4°C and 25 ± 2°C for different time periods on conversion of encapsulated protocorm of *C. bicolor* on B5 medium containing BA (4.42 µM).

Storage temperature (°C)	Storage duration (days)	Regeneration response (%)	No of shoots/bead	Shoot length (cm)/bead	No of Roots	Root Length (cm)
4°C	0	80	30.25±0.11[a]	2.58±0.74 [a]	1.11±0.43 [a]	1.15±0.13 [a]
	30	10.23	19.12±0.71[e]	2.40±0.56 [a]	1.31 ±0.50 [a]	1.00±0.90 [b]
	60			**Complete death**		
25°C	30	80	30.12±0.45 [a]	2.29±0.97 [a]	1.00±0.11 [a]	1.00±0.11 [a]
	60	75	27.65±0.98 [b]	1.95±0.62 [b]	0.95±0.98 [b]	0.95±0.98 [b]
	90	70	25.83±0.71 [c]	1.14±0.74 [b]	0.94±0.43 [b]	0.94±0.43 [b]
	180	60	20.59±0.60 [d]	0.83±0.54 [d]	0.53±0.81 [b]	0.53±0.81 [b]
	360	52	18.87±0.44 [e]	0.32±0.12 [e]	0.45±0.77 [c]	0.45±0.77 [c]

Values represent mean±SE. Each treatment was repeated twice and each treatment counted of 5 replicate culture tubes, each containing five encapsulated protocorms. Means in a column with the different letter (superscript) are significantly different according to DMRT ($p< 0.05$).

ilarly, 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/ regeneration (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 regeneration efficiency and the number of seedling/shoots 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±0.11 to 19. 12±0.71 while 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 shoots/bead, whereas, capsules stored at 4°C lost their viability completely after 30 days. Similarly, in relation to morphological changes, the numbers of shoots, root numbers, shoot and rootlength of thestored 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.1h and i) this procedure subsequently resulted in *in vitro* hardening of the plants. Acclimatization of plants grown in *in vitro* to *ex vitro* 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.

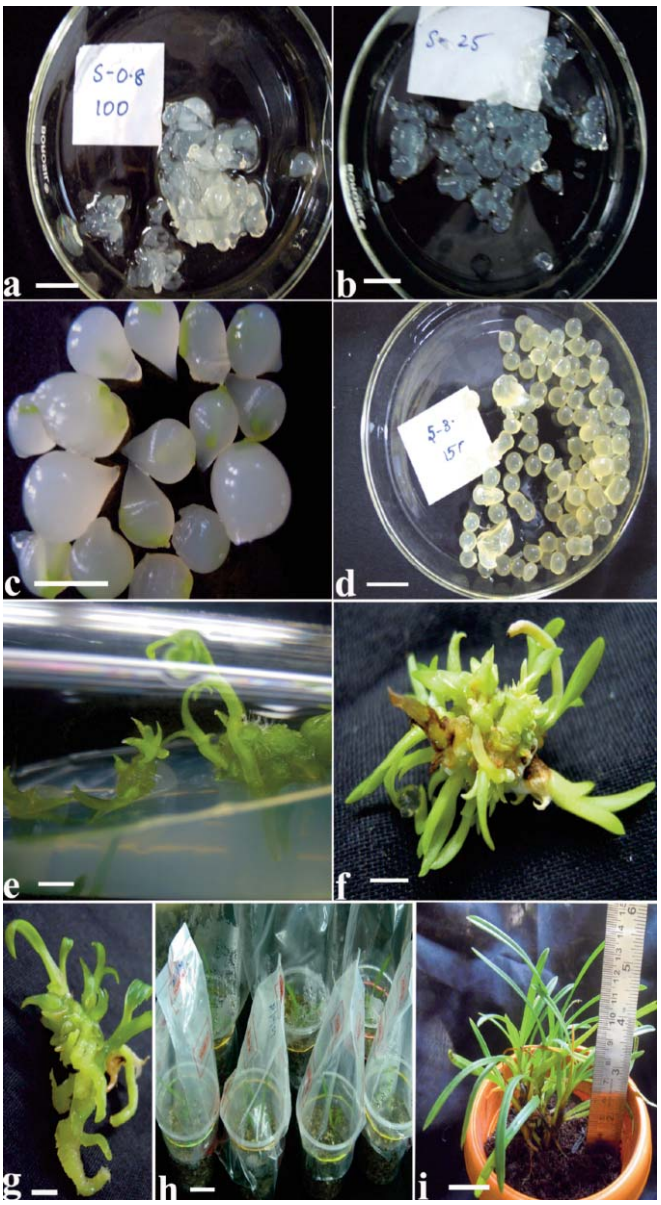

Fig. 1. Alginate-encapsulation, short-term storage and regeneration of *C. bicolor* using seed derived protocorm.

a. Beads prepared with 2% sodium alginate in75 mM calcium chloride solution. (Bar = 2 cm)
b. Beads prepared with 3% alginate in 75mM calcium chloride solution. (Bar = 2 cm)
c. Firm beads at 3 % sodium alginate in 100 mM $CaCl_2$ solution (Bar = 2 cm)
d. Hard brads 5 % at sodium alginate in 100 mM $CaCl_2$ solution. (Bar = 2 cm)
e. Conversion of synthetic seed & formation of multiple shoot/ seedling on B5 medium containing BA (4.42 µM). (Bar = 2 cm)
f. Conversion of synthetic seed & formation of multiple protocorm/ seedling on B5 medium containing Kn (13.92 µM). (Bar = 2 cm)
g. plantlets (Bar = 1cm)
h. *In vitro* hardening. (Bar = 2 cm)
i. Plantlet transferred to pot containing vermiculite cori peat (1:1). (Bar = 2 cm)

CONCLUSION

In conclusion, this study developed highly effective techniquesfor synthetic seed production, short-term conservation and regeneration of plantlets. The synthetic seed development protocol illustrated here offers a substitute scheme for mass propagation and germplasm distribution of this important orchid to laboratories and extension centers in distant places. Exultant plant recovery from encapsulated shoot tips following room temperature storage for more than 360 days indicates that the technique described in this report can be potentially exploited for short-term storage with the retention of genetic uniformity.

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Literature Cited

Adriani, M., Piccioni, E. and Standardi, A. 2000. Effect of different treatments on the conversion of 'Hayward' kiwifruit synthetic seeds to whole plants following encapsulation of *in vitro* derived buds. New Zeal. Journal of Crop and Horticultural Science, 28:59–67.

Ahmad, N. and Anis, M. 2010. Direct plant regeneration from encapsulated nodal segments of *Vitex negundo*. Biologia Plantarum, 54:748–752.

Alatar, A. and Faisal, M. 2012. Encapsulation of Rauvolfia tetraphylla microshoots as artificial seeds and evaluation of genetic fidelity using RAPD and ISSR markers. Journal of Medicinal Plants Research, 6:1367–1374.

Begum, A.A., Tamaki, M. and Kako, S. 1994. Formation of protocorm-like bodies (PLBs) and shoot development through *in vitro* culture of outer tissue of *Cymbidium* PLB. Journal of the Japanese Society for Horticultural Science, 63:663–673.

Bustam, S., Sinniah, U.R., Kadir, M.A., Zaman, F.Q. and Subramaniam, S. 2013. Selection of optimal stage for protocorm-like bodies and production of artificial seeds for direct regeneration on different media and short term storage of *Dendrobium* Shavin White. Plant Growth Regulators, 69: 215- 224.

Chand, S. and Singh, A. K. 2004. Plant regeneration from encapsulated nodal segments of *Dalbergia sissoo* Roxb., a timber yielding leguminous tree species. Journal of Plant Physiology, 161:237–243.

Chang, C. and Chang, W. C. 1998. Plant regeneration from callus of *Cymbidium ensifolium* var. misericors. Plant Cell Reports, 17:251-255.

Chugh, S., Guha, S. and Usha Rao, I. 2009. Micropropagation of orchids: A review on the potential of different explants. Scientia Horticulturae, 22:507–520.

Chung, J.D., Chun, C.K., and Choi, S.O. 1985. Asymbiotic germination of *Cymbidium ensifolium* II. Effects of several supplements to the medium, pH values and light and/or dark culture periods on growth of rhizome and organogenesis for rhizome. Journal of the Korean Society for Horticultural Science, 26:86–92.

Corrie, S. and Tandon, P. 1993. Propagation of *Cymbidium gianteum* wall through high frequency conversion of encapsulated protocorms under in vivo and *in vitro* conditions. Indian Journal of Experimental Biology, 31:61–64.

Danso, K.E. and Ford-Lyoyd, B.V. 2003. Encapsulation of nodal cuttings and shoot tips for storage and exchange of cassava germplasm. Plant Cell Reports, 21:718–725.

Das, M.C., Kumaria, S. and Tandon, P. 2011. Storage and high conversion frequency of encapsulated protocorm-like bodies of *Cymbidium devonianum* (orchid). Journal of Horticultural Science and Biotechnology, 86: 611-615.

Datta, K.B., Kanjilal, B. and Sarker, D. 1999. Artificial seed technology. Development of a protocol in *Geodorum densiflorum* (Lam.) Schltr. An endangered orchid. Current Science, 76:1142-1145.

Deb, C.R. and Pongener, A. 2011. Asymbiotic seed germination and *in vitro* seedling development of *Cymbidium aloifolium* (L.) Sw.: a multipurpose orchid. Journal of Plant Biochemistry and Biotechnology, 20:90–95.

Divakaran, M., NirmalBabu, K. and Peter, K.V. 2006. Conservation of *Vanilla* species, *in vitro*. Scientia Horticulturae, 110:175–180.

Gangopadhyay, G., Bandyopadhyay, T., Ramit, P., Gangopadhyay, S.B. and Mukherjee, K.K. 2005. Encapsulation of pineapple microshoots in alginate beads for temporary storage. Current Science, 88:972–977.

Gantait, S., Bustam, S. and Sinniah, U.R. 2012. Alginate-encapsulation, short-term storage and plant regeneration from protocorm-like bodies of *Aranda* Wan Chark Kuan 'Blue'× *Vanda coerulea* Grifft. ex. Lindl. (Orchidaceae). Plant Growth Regulation, 67:257–270.

Gantait, S. and Sinniah, U.R. 2012. Storability, post-storage conversion and genetic stability assessment of alginate-encapsulated shoot tips of monopodial orchid hybrid *Aranda* Wan CharkKuan 'Blue' 3 *Vanda coerulea* Grifft.ex. Lindl. Plant Biotechnology Reports, 7: 257- 266.

Ghosh, B. and Sen, S. 1994. Plant regeneration from alginate encapsulated somatic embryos of *Asparagus cooperi* Baker. Plant Cell Reports, 13:381–385.

Hoque, M.I., Roy, A.R., Sarker, R.H. and Haque, M. M. 1994. Micropropagation of *Cymbidium bicolor* through *in vitro* culture. Plant Tissue Culture, 4:45–51.

Hossain, M.M., Sharma, M., Teixeira Da Silva, J.A. and Pathak, P. 2010. Seed germination and tissue culture of *Cymbidium giganteum* Wall. ex Lindl. Scientia Horticulturae,123:479–487.

Huan, L.V.T. and Tanaka, M. 2004. Callus induction from protocorm-like body segments and plant regeneration in *Cymbidium* (Orchidaceae). Journal of Horticultural Science and Biotechnology, 79:406–410.

Hung, C.D. and Trueman, S.J. 2011. Encapsulation technology for short term preservation and germplasm distribution of the African mahogany *Khaya senegalensis*. Plant Cell, Tissue and Organ Culture, 107:397–405.

Hung, C.D. and Trueman, S.J. 2012a. Alginate encapsulation of shoot tips and nodal segments for short-term storage and distribution of the eucalypt *Corymbia torelliana* × *C. citriodora*. Acta Physiologiae Plantarum, 34:117–128.

Hung, C.D. and Trueman, S.J.P. 2012b. Reservation of encapsulated shoot tips and nodes of the tropical hardwoods *Corymbiatorelliana* × *C. citriodora* and *Khaya senegalensis*. Plant Cell, Tissue and Organ Culture, 109:341–352.

Khoddamzadeh, A.A., Sinniah, U.R., Kadir, M.A., Kadzimin, S.B., Mahmood, M. and Subramaniam, S. 2011. Establishment of a short-term storage method via encapsulation of protocorm-like bodies in *Phalaenopsis bellina* (Rchb.f.) Christenson. Seed Science and Technology, 39:697-702.

Khor, E., Ng, W.F. and Loh, C.S. 1998. Two-coat system for encapsulation of *Spathoglottis plicata* (Orchidaceae) seeds and protocorms. Biotechnology and Bioengineering, 59:635-639.

Larkin, P.J., Davies, P.A. and Tanner, G.J. 1998. Nurse culture of low number of *Medicago* and *Nicotiana* protoplasts using calcium alginate beads. Plant Science, 58:203–210.

Lata, H., Chandra, S., Khan, I.A. and El-Sohly, M.A. 2009. Propagation through alginate encapsulation of axillary buds of *Cannabis sativa* L. an important medicinal plant. Physiology and Molecular Biology of Plants, 15:79–86.

Lata, H., Chandra, S., Mehmedic, Z., Khan, I.A. and El-Sohly, M. A. 2012. *In vitro* germplasm conservation of high D9-tetrahydrocannabinol yielding elite clones of *Cannabis sativa* L. under slow growth conditions. Acta Physiologiae Plantarum, 34:743–750.

Lindemann, E.G.P., Gunckel, J.E. and Davidson, O.W. 1970. Meristem culture of *Cattleya*. American Orchid Society Bulletin, 39:1002-1004.

Mahendran, G., Muniappan, V., Ashwini, M., Muthukumar, T. and Narmatha Bai, V. 2013. Asymbiotic seed germination of *Cymbidium bicolor* Lindl. (Orchidaceae) and the influence of mycorrhizal fungus on seedling development. Acta Physiologiae Plantarum, 35:829–840.

Mahendran, G. and Narmatha Bai, V. 2012. Direct somatic embryogenesis and plant regeneration from seed derived protocorms of *Cymbidium bicolor* Lindl. Scientia Horticulturae, 135:40–44.

Malabadi, R.B., Teixeira da Silva, J.A., Nataraj, K. and Mulgund, G.S. 2008. Shoot tip transverse thin cell layers and 2, 4-epibrassinolide in the micropropagation of *Cymbidium bicolor* Lindl. Floriculture and Ornamental Biotechnology, 2: 44–48.

Mandal, J., Pattnaik, S. and Chand, P.K. 2000. Alginate encapsulation of axillary buds of *Ocimum americanum* L. (Hoary basil), *O. basilicum* (sweet basil), *O. gratissium* (shrubby basil) and

O. sanctum (sacred basil). *In Vitro* Cellular and Developmental Biology- Plant, 36:287–92.
Martin, K.P. 2003. Clonal propagation, encapsulation and reintroduction of *Ipsea malabarica* (Reichb.f) Hook JD, An endangered orchid. *In Vitro* Cellular and Developmental Biology- Plant, 39:322-326
Micheli, M., Hafiz, I.A. and Standardi, A. 2007. Encapsulation of *in vitro* derived explants of olive (*Oleaeuropaea* L. cv. Moraiolo) II. Effects of storage on capsule and derived shoots performance. Scientia Horticulturae, 113:286–292.
Mishra, J., Singh, M., Palni, L.M.S. and Nandi, S.K. 2011. Assessment of genetic fidelity of encapsulated microshoots of *Picrorhiza kurrooa*. Plant Cell Tissue Organ Culture, 104:181–186.
Mohanraj, R., Ananthan, R. and Narmatha Bai, V. 2009. Production and storage of synthetic seeds in *Coelogyne breviscapa* Lindl. Asian Journal of Biotechnology, 1:124–128.
Mohanty, P., Nongkling, P., Das, M.C., Kumaria, S. and Tandon, P. 2012. Short-term storage of alginate-encapsulated protocorm-like bodies of *Dendrobium nobile* Lindl.: an endangered medicinal orchid from North-east India. 3 Biotech, 3 (3): 235- 239.
Morel, G. 1964. Tissue culture—a new means of clonal propagation of orchids. American Orchid Society Bulletin, 33:437–478.
Nagananda, G.S., Nalini, S. and Rajath, S. 2011. Regeneration of encapsulated protocorm like bodies of medicinally important vulnerable orchid *Flickingeria nodosa* (Dalz.) Seidenf. International Journal of Botany, 7:301-313.
Nakashimada, Y., Uozumi, N. and Kobayashi, T. 1995. Production of plantlets for use as artificial seeds from horse radish hairy roots fragmented in a blender. Journal of Fermentation and Bioengineering, 79:458–464.
Nayak, N.R., Rath, S.P. and Patnaik, S. 1997. *In vitro* propagation of three epiphytic orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. And *Dendrobium moschatum* (Buch-Ham) Sw. through thidiazuron-induced high frequency shoot proliferation. Scientia Horticulturae, 71:243–250.
Nhut, D.T., Tien, T.N.T., Huong, M.T.N., Hien, N.T.H., Huyen, P.X., Luan, V.Q., Le, B.V. and Teixeira da Silva, J.A. 2005. Artificial seeds for preservation and propagation of *Cymbidium* spp. Propagation of Ornamental Plants, 5: 67–73.
Ozudogru, E.A., Kirdok, E., Kaya, E., Capuana, M., De Carlo, A. and Engelmann, F. 2011.Medium-term conservation of redwood [*Sequoia sempervirens* (D. Don) Endl.] *in vitro* shoot cultures and encapsulated buds. Scientia Horticulturae, 127:431–435.
Piccioni, E. and Standardi, A. 1995. Encapsulation of micropropagated buds of six woody species. Plant Cell Tissue Organ Culture, 42:221–226.
Rai, M.K., Asthana, P., Singh, S.K., Jaiswal, V.S. and Jaiswal, U. 2009. The encapsulation technology in fruit plants a review. Biotechnology Advance, 27:671–679.
Rai, M.K., Jaiswal, V.S. and Jaiswal, U. 2008. Encapsulation of shoot tips of guava (*Psidium guajava* L.) for short-term storage and germplasm exchange. Scientia Horticulturae, 118:33–38.
Reddy, M.C., Murthy, K.S.R. and Pullaiah, T. 2012. Synthetic seeds: A review in agriculture and forestry. African Journal of Biotechnology, 11:14254–14275.
Redenbaugh, K., Slade, D., Viss, P. and Fujii, J.A.A. 1987. Synthetic seed technology for mass cloning of crop plants: problems and perspectives. Horticultural Science, 22:796–814.
Rihan, H.Z., Al-Issawi, M., Burchett, S. and Fuller, M.P. 2011. Encapsulation of cauliflower (*Brassica oleracea* var. *botrytis*) microshoots as artificial seeds and their conversion and growth in commercial substrates. Plant Cell Tissue Organ Culture, 107:243–250.
Saiprasad, G.V.S. and Polisetty, R. 2003. Propagation of three orchid genera using encapsulated protocorm-like bodies. *In vitro* Cellular and Developmental Biology- Plant, 39: 42–48.
Sarmah, D.K., Borthakur, M. and Borua, P.K. 2010. Artificial seed production from encapsulated PLBs regenerated from leaf base of *Vanda coerulea* Grifft. ex. Lindl. – An endangered orchid.

Current Science, 98:686–690.
Sharma, S. and Shahzad, A. 2012. Encapsulation technology for short-term storage and conservation of a woody climber, *Decalepis hamiltonii* Wight and Arn. Plant Cell Tissue Organ Culture, 111:191–198.
Sharma, S., Shahzad, A., Jan, N. and Sahai, A. 2009a. *In vitro* studies on shoot regeneration through various explants and alginate-encapsulated nodal segments of *Spilanthes mauritiana* DC., an endangered medicinal herb. International Journal of Plant Developmental Biology, 3:56–61.
Sharma, S., Shahzad, A. and Sahai, A. 2009b. Artificial seeds for propagation and preservation of *Spilanthes acmella* (L.) Murr., a threatened pesticidal plant species. International Journal of Plant Developmental Biology, 3:62–64.
Sharma, S., Shahzad, A. and Teixeira da Silva, J.A. 2013.Synseed technology a complete synthesis. Biotechnology Advance, 31:186–207.
Sharma, A., Tandon, P. and Kumar, A. 1992. Regeneration of *Dendrobium wardianum* Warner (Orchidaceae) from synthetic seeds. Indian Journal of Experimental Biology, 30: 747-748.
Shimasaki, K. and Uemoto, S. 1990. Micropropagation of a terrestrial *Cymbidium* species using rhizomes developed from seeds and pseudo bulbs. Plant Cell Tissue Organ Culture, 22:237–244.
Shrivastava, V., Khan, S.A. and Banerjee, S. 2009. An evaluation of genetic fidelity of encapsulated microshoots of the medicinal plant: *Cineraria maritima* following 6 months of storage. Plant Cell Tissue Organ Culture, 99:193–198.
Singh, S.K., Rai, M.K., Asthana, P., Pandey, S., Jaiswal, V.S. and Jaiswal, U. 2009. Plant regeneration from alginate-encapsulated shoot tips of *Spilanthes acmella* (L.) Murr, a medicinally important and herbal pesticidal plant species. Acta Physiologiae Plantarum, 31:649–653.
Singh, S.K., Rai, M.K., Asthana, P. and Sahoo, L. 2010. Alginate-encapsulation of nodal segments for propagation, short-term conservation and germplasm exchange and distribution of *Eclipta alba* (L.). Acta Physiologiae Plantarum, 32: 607–610.
Sundararaj, S.G., Agrawal, A. and Tyagi, R.K. 2010. Encapsulation for *in vitro* short term storage and exchange of ginger (Zingiber officinale Rosc.) germplasm. Scientia Horticulturae, 125:761–766.
Swamy, M.K., Balasubramanya, S. and Anuradha, M. 2009. Germplasm conservation of patchouli (*Pogostemon cablin* Benth.) by encapsulation of *in vitro* derived nodal segments. International Journal of Biodiversity and Conservation, 1:224–230.
Tabassum, B., Nasir, I.A., Farooq, A.M., Rehman, Z., Latif, Z. and Husain, T. 2010. Viability assessment of *in vitro* produced synthetic seeds of cucumber. African Journal of Biotechnology, 9:7026–7032.
Teixeira da Silva, J.A., Giang, D.T.T., Chan, M.T., SanjayaNorikane, A., Chai, M.L., Chico-Ruız, J., Penna, S., Granstrom, T. and Tanaka, M. 2007. The influence of different carbon sources, photohetero-, photoauto- and photo mixotrophic conditions on protocorm-like body organogenesis and callus formation in thin cell layer culture of hybrid *Cymbidium* (Orchidaceae). Orchid Science and Biotechnology, 1:15–23.
Uozumi, N., Nakashimada, K.Y. and Kobayashi, T. 1992. Production of artificial seed from horseradish hairy root. Journal of Fermentation and Bioengineering, 74:21–26.
Vij, S.P., Kaur, P. and Gupta, A. 2001. Synseeds and their utility in orchids: *Dendrobium densiflorum* Lindl. Phytomorphology, 51:159–165.
Wang, X. 1988. Tissue culture of *Cymbidium*: plant and flower induction *in vitro*. Lindleyana 3:184–189.
Zhang, Y.F. and Yan, S. 2011. Factors affecting germination and propagators of artificial seeds of *Dendrobium candidum*. International Conference on Agricultural and Biosystems Engineering. Advances in Biomedical Engineering 1-2: 404-410.