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# Alginate-encapsulation, Short-term Storage and Plantlet Regeneration from Encapsulated Protocorm-like Bodies (PLBs) of *Cymbidium finlaysonianum* Lindl.: An Endangered Orchid of Thailand

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**Abstract**

Synthetic seed technology is becoming popular due to its wide application in germplasm conservation and for exchanges among countries in the floriculture trade. This method was used to study the bead formations and the conversion capabilities of *Cymbidium finlaysonianum* Lindl. For artificial seeds, new protocorm-like bodies (PLBs) (about 4 - 5 mm in diameter) of *Cymbidium finlaysonianum* Lindl. were isolated individually from 2-month-old proliferating PLB-clusters cultured in Vacin and Went (VW) liquid medium supplemented with 8.84 µM 6-benzyl-aminopurine (BAP). Different concentrations of sodium alginate (2, 3, 4 and 5 %) and calcium chloride ($CaCl_2.2H_2O$) (75, 85 and 100 mM) were tested to prepare bead formation. The ideal bead was obtained using 3 % sodium alginate and 100 mM $CaCl_2$ for 30 min, resulting in uniform spherical beads. Effects of different media (full-strength MS, ½ MS or ¼ MS liquid medium with 3 % (w/v) sucrose) in gelling matrix on artificial seed germination were explored. Of the various gelling matrices for germination, the ¼ MS liquid medium was the most effective in terms of germination percentage (90 %) and germination date (28 days). Germination of the artificial seeds was observed within 28 - 56 days of formation on various gelling matrices. The effects of different storage conditions and intervals on conversion (into plantlets with shoot and root) ability of artificial seeds were determined. Among the 4 temperature regimes of storage (0 ± 2, 4 ± 2, 8 ± 2 and 25 ± 2 °C), artificial seeds were successfully stored at 8 ± 2 °C, till 105 days showed percentage of conversion frequency at 44.0 % when cultured on a VW medium supplemented with 2 % (w/v) sucrose with 0.2 % (w/v) activated charcoal. The complete plantlets derived from germinated-encapsulated PLBs showed 90 % survival rate during their gradual acclimatization to greenhouse conditions after transferal of rooted plantlets to sterilized coconut husks and irrigation with water twice a day for 3 months under 70 - 80 % relative humidity and about 12 h photoperiod. The highest percentage of plantlet survival recorded at 12 months after reestablishment in the forest was 75.8 %.

**Keywords:** *Cymbidium finlaysonianum*, germplasm conservation, sodium alginate, short-term storage, encapsulation

## Introduction

Synthetic seed production, establishment of gene bank for *ex situ* conservation of plant germplasm in the form of field gene bank, seed gene bank, *in vitro* collection, and cryogenically preserved tissues is a common practice [1]. Alginate encapsulation provides a viable approach for *in vitro* germplasm conservation as it combines the advantages of clonal multiplication with those of seed propagation and storage [2].

Synthetic seed production technology via alginate encapsulation is presently considered as an efficient choice for both propagation and short-term to mid-term storage, in a number of commercially important orchids [3,4]. This system provides a low-cost, high-volume propagation [3]. Other advantages of synthetic seeds include: easy handling all through storage and transportation, storage potential without any loss in viability and also upholding of clonal property of the regenerating plantlets [5]. Notably, alginate coat of encapsulated explants shield plant tissues from physical and environmental injury, reduces dehydration, and offers mechanical pressure to grip the explants inside the gel matrix during storage [2]. The use of PLBs for synthetic seed development in orchids has been proven to be the most efficient [3] over other organs due to its superior regenerative character. In this perspective, the intervention of synthetic seed technology could be handy for the exchange of germplasm of this elite hybrid between laboratories and flowering-potted plant industries because of the miniature bead size and due to easy handling of these structures. Throughout the past years, substantial efforts have been made for propagation and storage of PLBs via encapsulation for a number of commercial orchid hybrids, such as *Dendrobium* 'Sonia', *Oncidium* 'Gower Ramsay' [3] and orchid species *Cymbidium bicolor* [6], *Cattleya leopoldii* [3], *Coelogyne breviscapa* Lindl. [4], *Aranda* Wan Chark Kuan 'Blue' x *Vanda coerulea* Grifft. ex. Lindl. [7].

In view of the importance of *C. finlaysonianum* Lindl. and the advantages of the synthetic seed technology, the present research was intended to develop a competent protocol for synthetic seed production in *C. finlaysonianum* Lindl., storage and consequent plant regeneration, in order to facilitate distribution of propagation material, as well as, exchange of germplasm material. Consequently, the influence of different storage temperatures and duration on germination and conversion (into plantlets with shoots and roots) of the capsules was assessed.

## Materials and methods

### Effects of different concentrations of sodium alginate and calcium chloride on bead formation of *C. finlaysonianum* Lindl.

New PLBs (about 4 - 5 mm in diameter) of *C. finlaysonianum* Lindl. were isolated individually from 2-month-old proliferating PLB-clusters which had been cultured in Vacin and Went [8] liquid medium (VW) supplemented with 8.84 µM BAP (**Figure 1B**). Sodium alginate was used as a gelling agent and calcium chloride ($CaCl_2.2H_2O$) solution was used as a complexing agent. The pH of the gelling and complexing agents was adjusted to 5.0 prior to autoclaving for 15 min at 121 °C.

Individual PLBs were dipped and drenched in 2, 3, 4 and 5 % (w/v) sodium-alginate solution containing 2 % (w/v) sucrose in VW liquid medium without any plant growth regulator for 10 min. Aliquots of the alginate solution, each containing one PLB, were aseptically pipetted out and gently dropped individually with a Pasteur pipette into the $CaCl_2$ solution (75, 85 and 100 mM). The droplets were then allowed to polymerize for 30 min to harden the alginate beads. The resulting beads (7 - 8 mm in diameter) were washed in sterile distilled water 3 times to remove the traces of $CaCl_2$ and transferred to sterile filter paper in Petri dishes for 5 min under a laminar air-flow cabinet to eliminate excess water. The alginate beads were then called artificial seeds or synthetic seeds or encapsulated seeds. Different concentrations of sodium alginate (2, 3, 4 and 5 %) and calcium chloride (75, 85 and 100 mM) were tested to prepare bead formation. The different uniform beads (clearness, form and consistency) were recorded.

**Effects of different media in gelling matrix on artificial seed germination**

Individual PLBs were dipped and drenched in 3 % (w/v) sodium-alginate solution containing full-strength MS [9], ½ MS or ¼ MS liquid medium with 3 % (w/v) sucrose without any plant growth regulator for 10 min. Aliquots of the alginate solution, each containing one PLB, were aseptically pipetted out and gently dropped individually with a Pasteur pipette into 100 mM $CaCl_2$ solution. The droplets were then allowed to polymerize for 30 min to harden the alginate beads. The resulting beads (7 - 8 mm in diameter) were washed in sterile distilled water 3 times to remove the traces of $CaCl_2$ and transferred to sterile filter paper in Petri dishes for 5 min under a laminar air-flow cabinet to eliminate the excess of water.

Artificial seeds of *C. finlaysonianum* Lindl. were placed on sterile Petri dishes for their germination testing. The pH of MS medium was adjusted to 5.7 with 1 N KOH or 1 N HCl prior to autoclaving for 15 min at 121 °C. All cultures were maintained at 25 ± 2 °C under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density (PPFD). The frequency of germination (%) was determined after 56 days of culture and the number of days to germination was also recorded.

**Effects of different concentrations of sodium alginate and calcium chloride on germination of artificial seeds**

Individual PLBs were dipped and drenched in various concentrations (3, 4 and 5 %) of sodium-alginate solution containing ¼ MS liquid medium with 3 % (w/v) sucrose without any plant growth regulator for 10 min. Aliquots of the alginate solution, each containing one PLB, were aseptically pipetted out and gently dropped individually with a Pasteur pipette into 75, 85 and 100 mM $CaCl_2$ solution. The droplets containing a PLB were then allowed to polymerize for 30 min to harden the alginate beads. The resulting beads (7 - 8 mm in diameter) were washed in sterile distilled water 3 times to remove the traces of $CaCl_2$ and transferred to sterile filter paper in Petri dishes for 5 min under a laminar air-flow cabinet to eliminate the excess of water and thereafter transferred to culture bottles containing 25 ml of nutrient medium. All cultures were maintained at 25 ± 2 °C under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD. The frequency of germination (%) and germination date of artificial seeds were recorded.

**Effects of different storage conditions and intervals on conversion ability of artificial seeds**

Individual PLBs were dipped and drenched in 3 % (w/v) sodium-alginate solution containing ¼ MS liquid medium with 3 % (w/v) sucrose without any plant growth regulator for 10 min. Aliquots of the alginate solution, each containing one PLB, were aseptically pipetted out and gently dropped individually with a Pasteur pipette into 100 mM $CaCl_2$ solution. The droplets containing a PLB were then allowed to polymerize for 30 min to harden the alginate beads. The resulting beads (7 - 8 mm in diameter) were washed in sterile distilled water 3 times to remove the traces of $CaCl_2$ and transferred to sterile filter paper in Petri dishes for 5 min under a laminar air-flow cabinet to eliminate excess water. The artificial seeds were then placed in sterile Petri dishes (ten beads/plate), sealed with parafilm and in different shelves of a refrigerator at temperature of 0 ± 2, 4 ± 2, 8 ± 2 and 25 ± 2 °C to be stored for 15, 30, 45, 60, 75, 90, 105 and 120 days. The Petri dishes were incubated under dark conditions. About 30 beads from each set stored in each temperature regime were taken out and cultured on a VW medium supplemented with 2 % (w/v) sucrose with 0.2 % (w/v) activated charcoal (AC) every 15 days. The encapsulated PLBs grew out in the medium rupturing the beads and were maintained there for a development into complete plantlets. The frequencies of conversion (%) of artificial seeds were recorded every 15 days of culture.

The culture media were solidified with 0.75 % (w/v) agar-agar (commercial grade). The pH of VW was adjusted to 5.0 with 1 N KOH or 1 N HCl prior to autoclaving for 15 min at 121 °C. All cultures were maintained at 25 ± 2 °C under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}$ $s^{-1}$ PPFD.

**Transplantation of plantlets/greenhouse acclimatization and field establishment and reintroduction**

The complete plantlets derived from germinated-encapsulated PLBs after storing at 8 ± 2 °C for 105 days, were taken out from culture bottles and rinsed thoroughly with tap water to remove residual nutrients and agar from the plantlets without causing harm to roots. These plantlets were treated with 0.1 % fungicide (bavistin) solution for 5 min and again washed with sterile water. All plantlets were blot dried and then transplanted into plastic pots containing sterilized coconut husks. The plastic pots were covered by polyethylene bags. The seedlings were grown in the greenhouse with 70 - 80 % relative humidity and about 12 h photoperiod, 300 - 400 µmol $m^{-2}$ $s^{-1}$ PPFD (shaded sunlight) and 33 ± 1 °C to 30 ± 1 °C day/night temperatures. The young plants were sprayed with water twice a day for 3 months. The percentage of plantlet survival was recorded (12 months after reestablishment in the Rusamilae forest, Pattani province).

**Experimental design and statistical analysis**

All the experiments were conducted with a minimum of 25 replicates per treatment and the experiments were repeated 3 times. The results are expressed as mean ± SE of 3 experiments. Experiments were performed in a completely randomized design (CRD). The data were analyzed by ANOVA using SPSS version 17.5 and the mean values were separated using Duncan's multiple range test (DMRT) at 95 % level of confidence.

## Results and discussion

**Effects of different concentrations of sodium alginate and calcium chloride on bead formation of *C. finlaysonianum* Lindl.**

The formation of beads and the subsequent success of the encapsulation depend on the concentration of alginate and calcium chloride used. In this study, the alginate-beads containing PLBs (2-month-old) showed different morphology (clearness, form and consistency) based on the different concentrations of sodium alginate and calcium chloride used (**Figure 2**).

However, 3 % (w/v) sodium alginate solution upon complexion with 100 mM $CaCl_2$ solution for 30 min was found to be the optimum combination for proper hardening of beads *C. finlaysonianum* Lindl. (**Table 1** and **Figure 2**). The mean sizes of bulk beads achieved by this procedure were 7 - 8 mm in diameter. In our study, 3 % (w/v) sodium alginate solution and 100 mM calcium chloride solution presumably helped in optimal ion exchange between $Na^+$ and $Ca^{2+}$, producing firm, clear and isodiametric beads [7]. In addition to this, 3 % sodium alginate with 100 mM $CaCl_2$ produced the most uniform beads for handling (**Figure 2**). On the contrary, high concentrations of sodium alginate (4 - 5 %, ++++), artificial seeds were diametric but too hard.

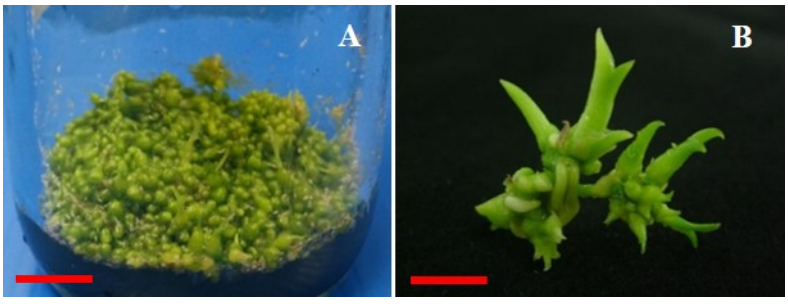


**Figure 1** *Cymbidium finlaysonianum* Lindl.: (A) asymbiotic germinated seeds derived from capsule after 90 days of culture on VW medium and (B) new 2-month-old PLBs derived from protocorms in VW liquid medium supplemented with 8.84 µM BAP. (Scale bar = 1 cm)

**Table 1** Synthetic seed formation of *C. finlaysonianum* Lindl. in different concentrations of sodium alginate and calcium chloride.

| Sodium alginate (w/v) | Calcium chloride (mM) | | |
|---|---|---|---|
| | 75 | 85 | 100 |
| 2 % | + | + | + |
| 3 % | ++ | ++ | +++ |
| 4 % | ++++ | ++++ | ++++ |
| 5 % | ++++ | ++++ | ++++ |

+ Abnormal bead (asymmetry), ++ Almost round, +++ Round (symmetry), ++++ Diametric and hard

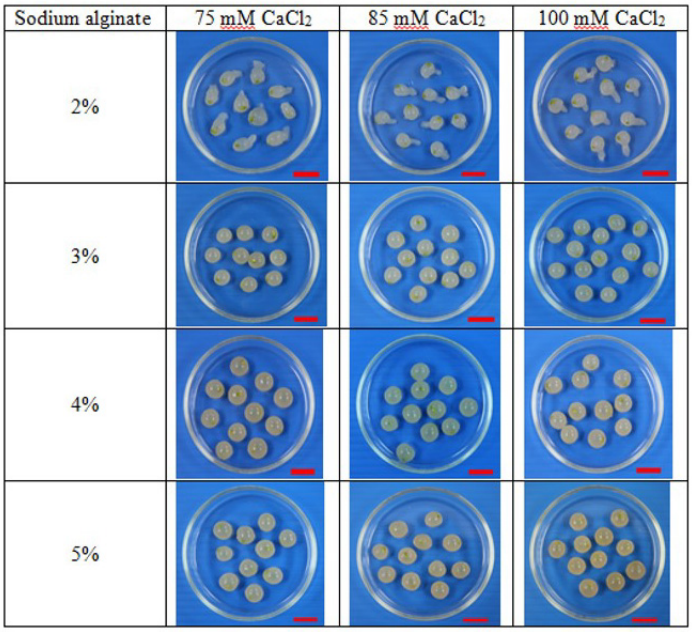


**Figure 2** Encapsulated PLBs of *C. finlaysonianum* Lindl. in different concentrations of sodium alginate and calcium chloride. (Scale bar = 1 cm).

The study developed efficient methods for alginate-encapsulation, germination and short-term storage of *C. finlaysonianum* Lindl. capsules for germplasm exchange between laboratories and distribution to nurseries. However, 3 % (w/v) sodium alginate solution upon complexion with 100 mM calcium chloride ($CaCl_2.2H_2O$) solution for 30 min was found to be the optimum combination for proper hardening of beads *C. finlaysonianum* Lindl. It was reported by several workers that 3 % sodium alginate with 100 mM $CaCl_2$ was the most ideal combination for synthetic seed production [10,11].

In most reports, 3 % (w/v) sodium alginate and 100 mM $CaCl_2$ for 20 - 30 min has proved to be the best combination for the formation of an ideal synthetic seed [7], [11-17]. However, 3 % sodium alginate upon complexion with 75 mM $CaCl_2$ for 20 - 30 min was found to be the optimum combination for proper hardening of beads of orchids, such as of *Dendrobium*, *Oncidium* and *Cattleya* orchids [3].

In contrast, for the encapsulation of nodal segments of medicinal plant, such as *Pogostemon cablin* [18], *Spilanthes acmella* [19] and the microshoots of *Zingiber officinale* [20], 4 % sodium alginate with 100 mM calcium chloride was optimum. This variation in sodium alginate concentration for synthetic

seed 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 [21] and Mandal *et al*. [22].

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 [23]. On the contrary, high concentrations of sodium alginate (5 - 6 %), beads were diametric but too hard, causing considerable delay in sprouting of shoots [7,12,19,24] which was also observed in our study (**Table 2** and **Figure 2**). Lower concentrations (2 %) of sodium alginate were not appropriate since the capsules were not in definite shape and excessively soft to grip.

**Effects of different media in gelling matrix on artificial seed germination**

Providing sufficient nutrients for the plant tissue. Of the various gelling matrix for germination test, ¼ MS liquid medium was the most effective in terms of germination percentage (90 %) and germination date (28 days) (**Figure 3**). Germination of the artificial seeds was observed within 28 - 56 days of formation on various gelling matrix. The lowest germination (30 %) was recorded in full-strength MS. The full-strength MS or ½ MS liquid medium in the gelling matrix might be disadvantageous owing to an overdose of the nutrient element content resulting in nutrient toxicity [7]. The germination occurred primarily by breaking open the calcium alginate matrix resulting in the emergence of shoots followed by gradual emergence of root. In the present study, artificial seeds of *C. finlaysonianum* Lindl. exhibited simultaneous production of shoots and roots without any specific root induction medium.

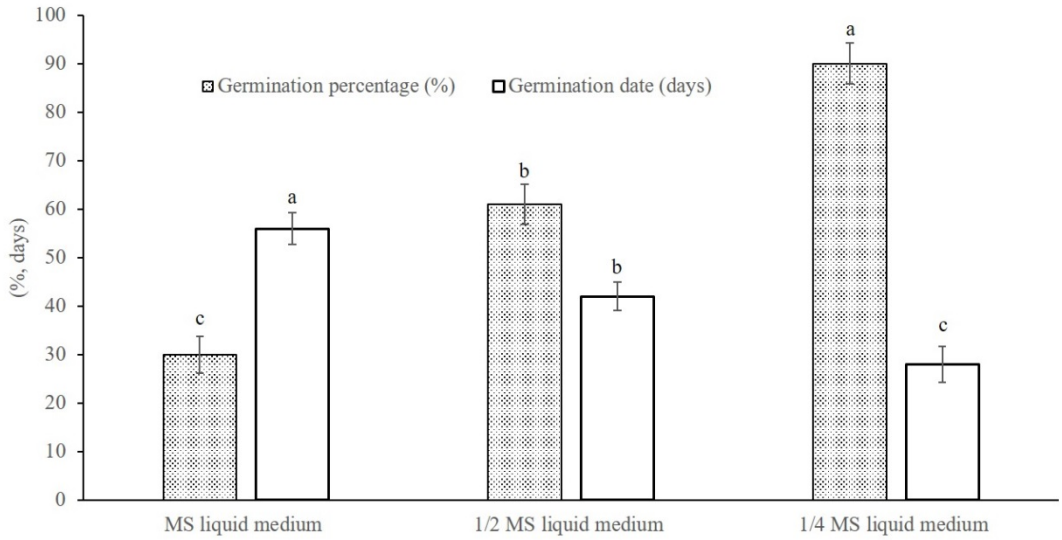


**Figure 3** The relationship between germination percentage and germination date of artificial seeds from 3 % (w/v) sodium alginate solution and 100 mM calcium chloride solution of encapsulated PLBs with gelling agent as MS liquid medium, ½ MS liquid medium and ¼ MS liquid medium. Data represent mean ± standard error of 25 replicate explants per treatment in 3 repeated experiments. Data for each *column* followed by different *alphabets* are significantly different according to Duncan's multiple range test at $p = 0.05$.

**Effects of different concentrations of sodium alginate and calcium chloride on germination of artificial seeds**

The days for germination and frequency of germination varied with change in the concentrations of sodium alginate and $CaCl_2$. Among the various concentrations of sodium alginate (3, 4 and 5 %) and $CaCl_2$ (75, 85 and 100 mM) used, PLBs encapsulated with 3 % sodium alginate and hardened in 100 mM $CaCl_2$ supported significantly higher frequency of synthetic seed germination (90 %). Here, the artificial seeds germinated within 28 days of culture. All other treatments resulted in lower germination frequency and more days for germination (**Table 2**).

At higher concentration of sodium alginate (4 and 5 %) the capsules were effectively firm to cause substantial delay in propagule emergence. There are several reports suggesting a direct relationship between higher frequency of germination of encapsulated somatic embryos with reduced exposure time to $CaCl_2$ [25,26]. This was mainly attributed to high concentrations or excessive exposure of the embryos to the complexing agent ($CaCl_2$) resulting in more absorption and penetration of $CaCl_2$ into the embryo, which can generate growth inhibition that is reflected in a decrease in the germination response and subsequent development in the field [25].

**Table 2** Effects of different concentrations of sodium alginate and calcium chloride on germination percentage after 42 days of culture and germination date of artificial seeds.

| Sodium alginate (%) | Calcium chloride (mM) | Germination percentage (%) | Germination date (days) |
|---|---|---|---|
| 3 | 75 | 82[b] | 27[c] |
| 3 | 85 | 81[b] | 28[c] |
| 3 | 100 | 90[a] | 28[c] |
| 4 | 75 | 73[c] | 35[b] |
| 4 | 85 | 72[c] | 34[b] |
| 4 | 100 | 72[c] | 35[b] |
| 5 | 75 | 65[d] | 40[a] |
| 5 | 85 | 64[d] | 40[a] |
| 5 | 100 | 60[d] | 42[a] |

Means followed by different letters within column are significantly different from others at 5 % level by DMRT.

**Effects of different storage conditions and intervals on conversion ability of artificial seeds**

*C. finlaysonianum* Lindl. PLBs (2-month-old) of size ranging from 4 - 5 mm in diameter were encapsulated as synthetic seeds prepared by alginate encapsulation, and then stored in artificial endosperm solution at 0 ± 2, 4 ± 2, 8 ± 2 and 25 ± 2 °C conditions in interaction with different storage intervals of 15, 30, 45, 60, 75, 90, 105 and 120 days to evaluate the comparative regrowth capacity of synthetic seeds.

In this study, among the 4 temperature regimes, storage temperature at 8 ± 2 °C gave promising results for seed germination. Conversion percentage of synthetic seeds decreased from 100.00 % to 44.00 % until 105 days of storage at 8 ± 2 °C under dark conditions. However, the encapsulated PLBs stored more than 105 days in such conditions gave no germination (**Table 3** and **Figure 4**). Encapsulated PLBs stored at 0 ± 2 and 4 ± 2 °C lost their viability completely (**Table 3**). At 25 ± 2 °C, the encapsulated PLBs germinated when storing in sterile Petri dishes under dark conditions (**Table 3** and **Figure 5**). At 120 days of storage, synthetic seeds that were stored in sterile Petri dishes at 8 ± 2 °C under dark conditions had dried up and were unable to germinate. It was observed that when the encapsulated PLBs were stored for 105 days, the encapsulated PLBs appeared yellow and unhealthy when they were first

taken out from the dark, but when the encapsulated PLBs were cultured onto VW medium, they gradually became green and looked healthier.

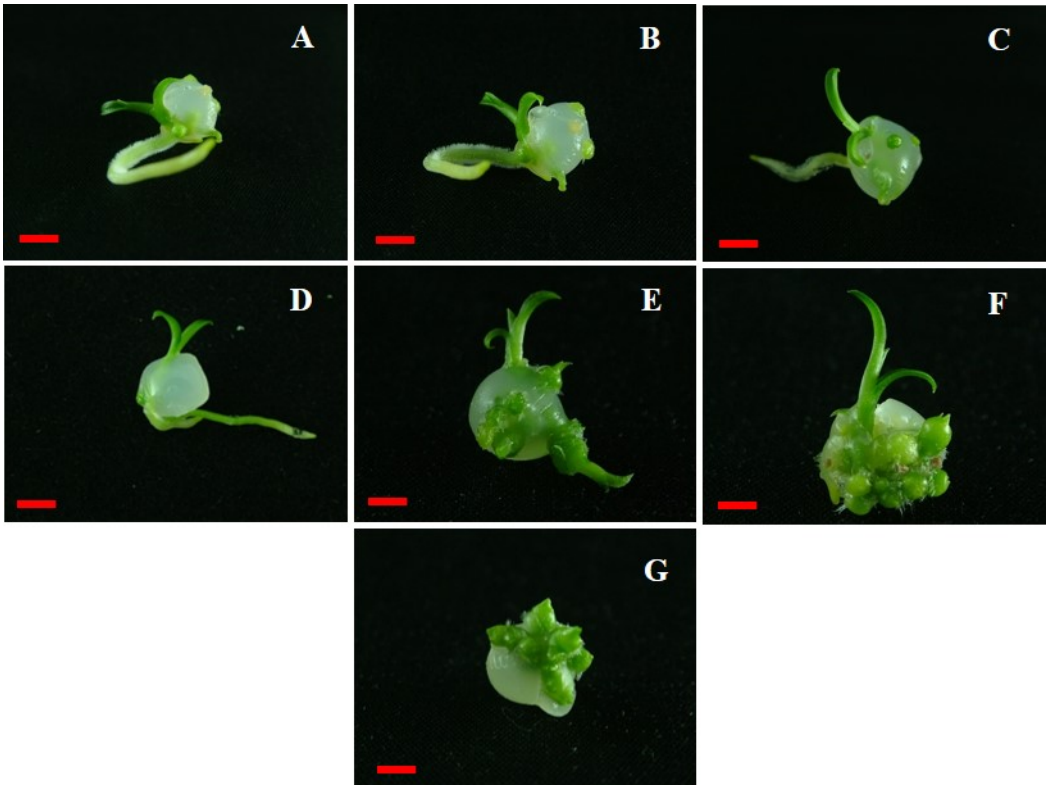


**Figure 4** Regeneration of encapsulated PLBs of *C. finlaysonianum* Lindl. on VW agar medium supplemented with 0.2 % (w/v) activated charcoal for 6 weeks: (A) after storing at 8 ± 2 °C for 15 days, (B) 30 days, (C) 45 days, (D) 60 days, (E) 75 days, (F) 90 days and (G) 105 days. (Scale bar = 1 cm).

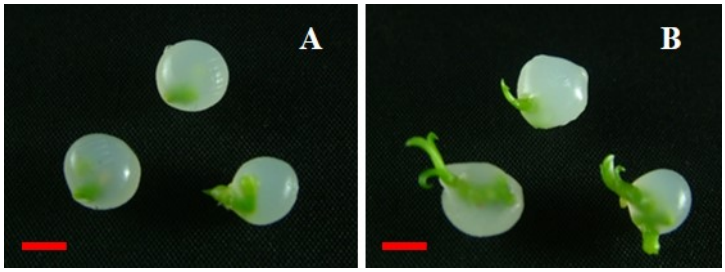


**Figure 5** Regeneration of encapsulated PLBs of *C. finlaysonianum* Lindl. on VW medium supplemented with 0.2 % (w/v) activated charcoal after storing at 25 ± 2 °C for 30 days: (A) 2 weeks of regeneration and (B) 4 weeks of regeneration. (Scale bar = 1 cm).

**Table 3** Effects of different storage temperatures and time on conversion of encapsulated PLBs of *C. finlaysonianum* Lindl.

| Storage temperature (°C) | Storage duration (days) | Conversion (Mean ± SE) (%) |
|---|---|---|
| 0 ± 2 °C | 15 | 0.00 ± 0.00[g] |
| | 30 | 0.00 ± 0.00[g] |
| | 45 | 0.00 ± 0.00[g] |
| | 60 | 0.00 ± 0.00[g] |
| | 75 | 0.00 ± 0.00[g] |
| | 90 | 0.00 ± 0.00[g] |
| | 105 | 0.00 ± 0.00[g] |
| | 120 | 0.00 ± 0.00[g] |
| 4 ± 2 °C | 15 | 0.00 ± 0.00[g] |
| | 30 | 0.00 ± 0.00[g] |
| | 45 | 0.00 ± 0.00[g] |
| | 60 | 0.00 ± 0.00[g] |
| | 75 | 0.00 ± 0.00[g] |
| | 90 | 0.00 ± 0.00[g] |
| | 105 | 0.00 ± 0.00[g] |
| | 120 | 0.00 ± 0.00[g] |
| 8 ± 2 °C | 15 | 100.00 ± 0.00[a] |
| | 30 | 86.67 ± 0.12[b] |
| | 45 | 73.53 ± 1.67[c] |
| | 60 | 60.00 ± 0.00[d] |
| | 75 | 52.00 ± 0.00[e] |
| | 90 | 50.00 ± 0.00[e] |
| | 105 | 44.00 ± 0.00[f] |
| | 120 | 0.00 ± 0.00[g] |
| 25 ± 2 °C | 15 | 100.00 ± 0.00[a] |
| | 30 | 100.00 ± 0.00[a] |
| | 45 | * |
| | 60 | * |
| | 75 | * |
| | 90 | * |
| | 105 | * |
| | 120 | * |

*Encapsulated PLBs germinated during storing in sterile Petri dishes
Means followed by different letters within column are significantly different from others at 5 % level by DMRT.

Saiprasad and Polisetty [3], Lambardi *et al.* [27] and Mohanraj *et al.* [4] observed that encapsulated PLBs in the *Dendrobium* and *Oncidium* orchid species could generally maintain maximum germination percentage when stored at 4 °C for 45 and 60 days. However, there is a decrease in the percentage of conversion to plantlets when the storage at low temperature is prolonged [27].

In this study, among the 4 temperature regimes, storage temperature at 8 ± 2 °C gave promising results for germination of capsules. Conversion percentage of synthetic seeds decreased from 100.00 % to 44.00 % until 105 days of storage at 8 ± 2 °C under dark conditions. However, the encapsulated PLBs stored more than 105 days in such conditions gave no germination at all. Encapsulated PLBs stored at

0 ± 2 °C and 4 ± 2 °C lost their viability completely due to the fluctuation in temperature and cold stress during the storage [34].

From the success of the present study, it seems that the storage of capsules in sterile Petri dishes and sealed with Parafilm (though having limited supply of oxygen for respiration of capsules) proved to be suitable to protect loss of humidity which is essential for retention of viability of encapsulated PLBs, since sodium alginate have been reported to succumb to rapid dehydration [28]. In addition, encapsulation of PLBs of this orchid appears to be a promising tool for storage and on-demand supply of plant material for propagation or germplasm exchange. Similar use of the encapsulation method for storage have also been reported earlier in many other endemic and endangered orchids like *Cymbidium bicolor* [6] and *Ipsea malabarica* [29]. The *in vitro* storage achieved for *C. finlaysonianum* Lindl. in our study has the prospective to cut the cost for maintaining the continuous proliferating PLB cultures, because of the abridged requirement for manual labor due to less frequent subculturing. According to Rai *et al*. [30], an important feature of the encapsulated vegetative propagules is their capability to retain viability after storage for a sufficient period required for exchange of germplasm.

**Transplantation of plantlets/greenhouse acclimatization and field establishment and reintroduction**

The complete plantlets derived from germinated-encapsulated PLBs showed 90 % survival rate during their gradual acclimatization to greenhouse conditions after transferring of rooted plantlets to sterilized coconut husks and irrigation with water twice a day for 3 months under 70 - 80 % relative humidity about 12 h photoperiod, 300 - 400 μmol $m^{-2}$ $s^{-1}$ PPFD (shaded sunlight) and 33 ± 1 to 30 ± 1 °C day/night temperature (**Figure 6A**). All the plants looked healthy. Plantlets without a well-developed root were unable to survive when transferred to greenhouse. The highest percentage of plantlet survival recorded at 12 months after reestablishment in the Rusamilae forest, Pattani province was 75.8 % (**Figure 6B**). The hardened plants did not show detectable variation in morphological or growth characteristics as compared to the field-grown parent plants.

Reintroduction of native species has become increasingly important in conservation worldwide for recovery of rare species and restoration purposes. However, some studies have reported the outcome of reintroduction efforts in plant species [31]. For example, Seeni and Latha [32] reported the ecorehabilitation of the endangered Blue Vanda (*Vanda coerulea*) and the survival rate was more than 70 %. Zeng *et al*. [33] reported reestablishment of lady's slipper orchid (*Paphiopedilum wardii* Sumerh.) in the alien forest habitat Ehuangzhang in which 65 % of reestablished seedlings survived. In this study, *C. finlaysonianum* Lindl. plantlets were successfully established in the field and reintroduced into the forest. The highest percentage of plantlet survival recorded at 24 months after reestablishment in the Rusamilae forest, Pattani province was 71.6 %, possibly because of suitable irradiation, soil, water, nutrients, environmental humidity and other factors in the former [33].

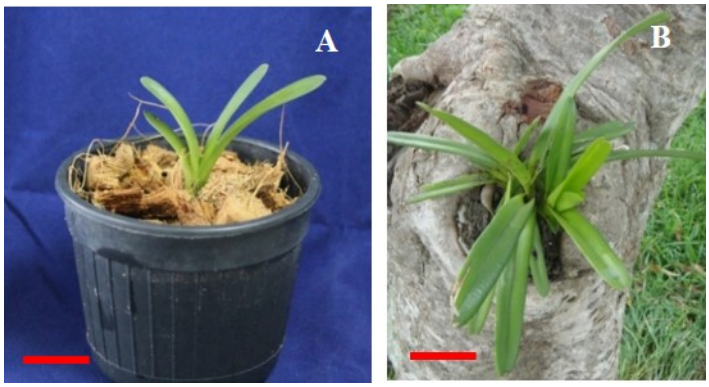


**Figure 6** (A) Healthy plants derived from germinated-encapsulated PLBs of *C. finlaysonianum* Lindl. with well expanded leaves in pots containing sterilized coconut husks after 3 months of transplanting and (B) establishment of *in vitro* plantlets in the wild 12 months after reestablishment in the Rusamilae forest, Pattani province. (Scale bar = 1 cm).

## Conclusions

In conclusion, this is the first report of synthetic seed production, storage and conversion of PLBs in *C. finlaysonianum* Lindl. This technique has not been explored in *C. finlaysonianum* Lindl. before. 1) Different concentrations of sodium alginate (2, 3, 4 and 5 %) and calcium chloride (75, 85 and 100 mM) were tested to prepare bead formation. The ideal bead was obtained using 3 % sodium alginate and 100 mM $CaCl_2$ for 30 min, resulting in uniform spherical beads. 2) Effects of different media (full-strength MS, ½ MS or ¼ MS liquid medium with 3 % (w/v) sucrose) in a gelling matrix on artificial seed germination. Of the various gelling matrix for germination test, ¼ MS liquid medium was the most effective in terms of germination percentage (90 %) and germination date (28 days). Germination of the artificial seeds was observed within 28 - 56 days of formation on various gelling matrix. 3) Effects of different storage conditions and intervals on conversion (into plantlets with shoot and root) ability of artificial seeds. Among the 4 temperature regimes of storage (0 ± 2, 4 ± 2, 8 ± 2 and 25 ± 2 °C), artificial seeds were successfully stored at 8 ± 2 °C, till 105 days showed percentage of conversion frequency at 44.0 % when culture on VW medium supplemented with 2 % (w/v) sucrose with 0.2 % (w/v) activated charcoal. 4) The complete plantlets derived from germinated-encapsulated PLBs showed 90 % survival rate during their gradual acclimatization to greenhouse conditions after transferring of rooted plantlets to sterilized coconut husks and irrigation with water twice a day for 3 months under 70 - 80 % relative humidity about 12 h photoperiod. The highest percentage of plantlet survival recorded at 12 months after reestablishment in the Rusamilae forest, Pattani province was 75.8 %.

It was also observed that plantlets from synthetic seeds were healthy, green and free from any morphological disorder. These results also confirm that synthetic seed technology is an authentic method for exchange of plantlets grown *in vitro* from one laboratory to another aseptically as well as without danger of any loss. Stock cultures of imported artificial seeds were maintained for clonal propagation.

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