Journal of Ornamental Plants
Available online on: www.jornamental.com
ISSN (Print): 2251-6433 ISSN (Online): 2251-6441

Effective Use of Synthetic Seed Technology in the Regeneration of *Dendrobium* White Fairy Orchid

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Received: 17 October 2013 Accepted: 13 February 2014
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Abstract

The synthetic seed technology is becoming popular due to its wide application in germplasm conservation and for exchanges between countries in the floriculture trade. In this study, this method was used to study the germination and conversion capabilities of orchid species *Dendrobium* White Fairy when stored at different storage times and with different storage containers. A high germination percentage of 80% was observed for encapsulated synthetic seeds up to 150 days of storage in Petri dishes and screw capped polypropylene tubes and this percentage only began to decline gradually from 150 storage days and more. Besides that, synthetic seeds stored in polypropylene tubes were shown to germinate faster and develop into plantlets with longer shoots and roots as compared to those stored in the Petri dishes. This proved the efficiency and suitability of polypropylene tubes over Petri dishes as storage containers.

Keywords: *Dendrobium*, Protocorm-like bodies, Regeneration, Synthetic seeds.

INTRODUCTION

In the orchid family, *Dendrobium* is one of the largest tropical genus that is found in diverse habitats of Asia, with about 1,200 species. Many of its hybrid species are relatively easy to grow and due to its wide variety, colourful nature of the flowers and its compact size for some species, it is very popular in the retail trade, and it is one of the most important orchids in the horticulture industry (Martin and Madassery, 2006). *Dendrobium* White Fairy, which is the target of our study, is a hybrid between *Dendrobium* Singapore White and *Dendrobium* Walter Oumae. This tropical orchid that is locally bred in Singapore has an average height of about 2 to 3 feet and blooms with about 6 to 12 buds in a star-shaped blossom and a gentle jasmine-like scent. Its elegant appearance made it very popular as ornamental plants as well as for landscaping purposes and they are widely exported as cut flowers.

In general, propagation of orchids from seeds is relatively difficult due to its minute seed size and lack of endosperm, heterozygousity of seeds and the requirement of the seed to associate with mycorrhizal fungi for germination (Saiprasad, 2001). The method used to germinate orchid seeds in the orchid growing industry is asymbiotic micropropagation, where seeds are germinated and cultured aseptically using culture media such as MS media (Murushige and Skoog, 1962) supplemented with a carbon source. Although this method is able to maintain the genetic uniformity of the plants, it is more suitable for small scale propagation as it is labour intensive and has relatively low multiplication rate (Saiprasad, 2001).

An alternative method, which has been studied and found to be more effective, is the synthetic seed technology. This method is preferred because it combines the advantages of clonal propagation and seed propagation with the possibility of long term storage of the seeds through encapsulation in a gel-like matrix (Lambardi *et al*., 2006). In this technique, non-embryogenic vegetative propagules such as shoot tips, nodal segments or axillary buds, protocorm-like bodies (PLBs) or calluses are artificially encapsulated using sodium alginate as the preferred coating agent. These synthetic hydrogel seeds contain nutrients that will help in the survival and speedy growth of the embryos into plantlets during their cultivation after storage (Sharma *et al.*, 2013). This cost effective method can be scaled up and has been shown to be successful in several commercially important agronomic and horticultural crops, such as bananas and potatoes (Ganapathi *et al.*, 1992; Nyende *et al.*, 2003), as well as ornamental plants such as lilac and orchids (Lambardi *et al.,* 2006). In orchids, PLBs are used for encapsulation and has been shown to successfully germinate and regenerate into plantlets (Ara *et al.,* 2000; Saiprasad and Polisetty, 2003; Gantait *et al.,* 2012).

In view of this technology, various optimizations have been performed and reported in orchid propagation using synthetic seeds, such as the concentration of sodium alginate and calcium chloride as gelling agents, the stage of PLBs suitable for encapsulation, the storage period and temperature of the synthetic seeds (Saiprasad and Polisetty, 2003; Mohanraj *et al.*, 2009; Gantait *et al.*, 2012). The objective of this study is to determine the effects of different storage times and compare between different storage containers on the germination and regeneration of *Dendrobium* White Fairy orchids using the synthetic seed technology.

MATERIALS AND METHODS

Chemicals, media and solutions

The following chemicals and media were used in this study.

Calcium chloride (MP Biomedicals, Cat. No. 153502); Sodium alginate (MP Biomedicals, Cat. No. 218295); Murashige & Skoog (MS) Basal media (PhytoTechnology Laboratories, Cat. No. M519); Phyto Agar (plantMedia, Cat. No. 40100072-2).

For the preparation of encapsulation matrix, 3% (w/v) sodium alginate was prepared in ½ MS liquid media containing 2% sucrose, while 75 mM calcium chloride ($CaCl_2.2H_2O$) was prepared in double distilled water.

Plant material, culture media and culture conditions

Green capsule seed pods of *Dendrobium* White Fairy orchid were obtained via self-pollination. The seeds were germinated aseptically in Magenta GA-7™ vessels with 50 mL of ½ MS media supplemented with 15% (v/v) coconut water, 2% (w/v) sucrose and 0.6% (w/v) Phyto-agar (PlantMedia). All media were adjusted to pH 5.2 before autoclaving at 121°C for 20 min. All cultures were incubated and maintained at 25±1°C with a 24-hour photoperiod.

After 4 weeks of cultivation in solid media, the green pin-head-like PLBs formed were then transferred to ½ MS liquid media supplemented with 15% (v/v) coconut water and 2% (w/v) sucrose and maintained on a rotary shaker at 200 rpm under the above specified conditions for PLB induction. PLBs in the liquid media were subcultured every 3 weeks with fresh media until they were ready to be used for the experiments.

Encapsulation of *Dendrobium* White Fairy orchid PLBs

Twelve-week old *Dendrobium* White Fairy orchid PLBs were used in this study. The encapsulation procedure was adapted from Lambardi *et al.,* 2006. Encapsulation was carried out by mixing PLBs (about 3-4 mm diameter) with 3% (v/v) sodium alginate solution. Using a 7 mm diameter cut micropipette tip, aliquots of the alginate solution, each containing one PLB, were aseptically pipetted out and gently dropped into 75 mM $CaCl_2.2H_2O$ solution (Gantait *et al.*, 2012). The calcium alginate beads that were formed were maintained in the $CaCl_2.2H_2O$ solution for 30 min in a continuously stirring environment to prevent the beads from adhering to each other. These synthetic seeds were then decanted from the $CaCl_2.2H_2O$ solution, rinsed thrice in sterile water and blotted dry with sterilized filter paper.

Storage of encapsulated PLBs

The experiment aims to observe the effects of different storage time and compare between different storage containers on the germination and conversion ability of the encapsulated synthetic seeds. Non-encapsulated, or naked PLBs were used as the experimental control.

Storage time points of 0, 30, 60, 90, 120, 150 and 180 days were selected for this study. For each time point, three replicates were used and each replicate consists of a set of 40 synthetic seeds, which were prepared via encapsulation. These synthetic seeds were then divided equally into 2 groups and each group was stored in either a Petri dish or 15 ml polypropylene (PP) tube. Both storage containers contain 2 ml of sterile water. The same protocol was conducted for non-encapsulated PLB controls at 0, 30 and 60-day storage time-points. The control and the encapsulated synthetic seeds were then stored at 25±1oC under dark conditions until the storage time point is reached.

Assessment of germination and direct conversion of stored synthetic seeds

After each storage time point, the synthetic seeds were taken out of the Petri dish or 15 ml PP tube and were cultured aseptically in Petri dishes with 30 ml of ½ MS media containing 2% (w/v) sucrose and 0.6% (w/v) Phyto-agar (Plant Media). The cultures were incubated and maintained at 25±1°C with a 16-hour photoperiod. Germination of the seeds was monitored and recorded based on the number of days taken for the shoot to emerge from the gel matrix (Gantait *et al.*, 2012). The conversion of synthetic seed to plantlet was recorded after 50 days of in vitro culture. The percentage of germination and conversion into plantlet were then calculated and these results were compared to the controls.

RESULTS AND DISCUSSION

Encapsulation of *Dendrobium* White Fairy orchid PLBs

Dendrobium White Fairy orchid PLBs of size ranging from 3 to 4 mm in diameter (12

weeks old) were used because previous studies has shown that this size range is ideal for encapsulation and optimum conversion in orchids (Corrie and Tandon, 1993). PLBs smaller than the size range displayed poor conversion frequencies which could be due to tissue immaturity, thus the PLBs were unable to withstand encapsulation or require a longer time to emerge from the gelling matrix (Corrie and Tandon, 1993; Saiprasad and Polisetty, 2003; Poobathy *et al.*, 2009).

Besides that, Saiprasad (2001), Lambardi *et al.* (2006) and Gantait *et al*. (2012) documented that the ideal synthetic seeds were obtained using 3% sodium alginate and 75 mM calcium chloride as the gelling matrix. This combination creates optimal ion exchange between the sodium and calcium ions, which produces firm, clear and isodiametric beads (Gantait *et al.*, 2012). In addition, sodium arginate is also ideal because of its low toxicity to the embryo and the gel bead is able to protect the fragile embryo during handling (Ara *et al*., 2000). Germination was also best achieved when half-strength MS media was used in the gelling matrix, providing sufficient nutrients for the plant tissue (Gantait *et al.*, 2012). Based on the collated findings, the encapsulation protocol for *Dendrobium* White Fairy orchid PLBs was adapted (Fig. 1).

Assessment of germination and conversion of encapsulated PLBs

Based on studies by Saiprasad and Polisetty (2003), Lambardi *et al.* (2006) and Mohanraj *et al.* (2009), it was 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 conversion percentage to plantlets when the storage at low temperature is prolonged (Lambardi *et al.*, 2006). In a recent paper, it was found that storage at 25°C showed a higher percentage in germination and in conversion to plantlets as compared to

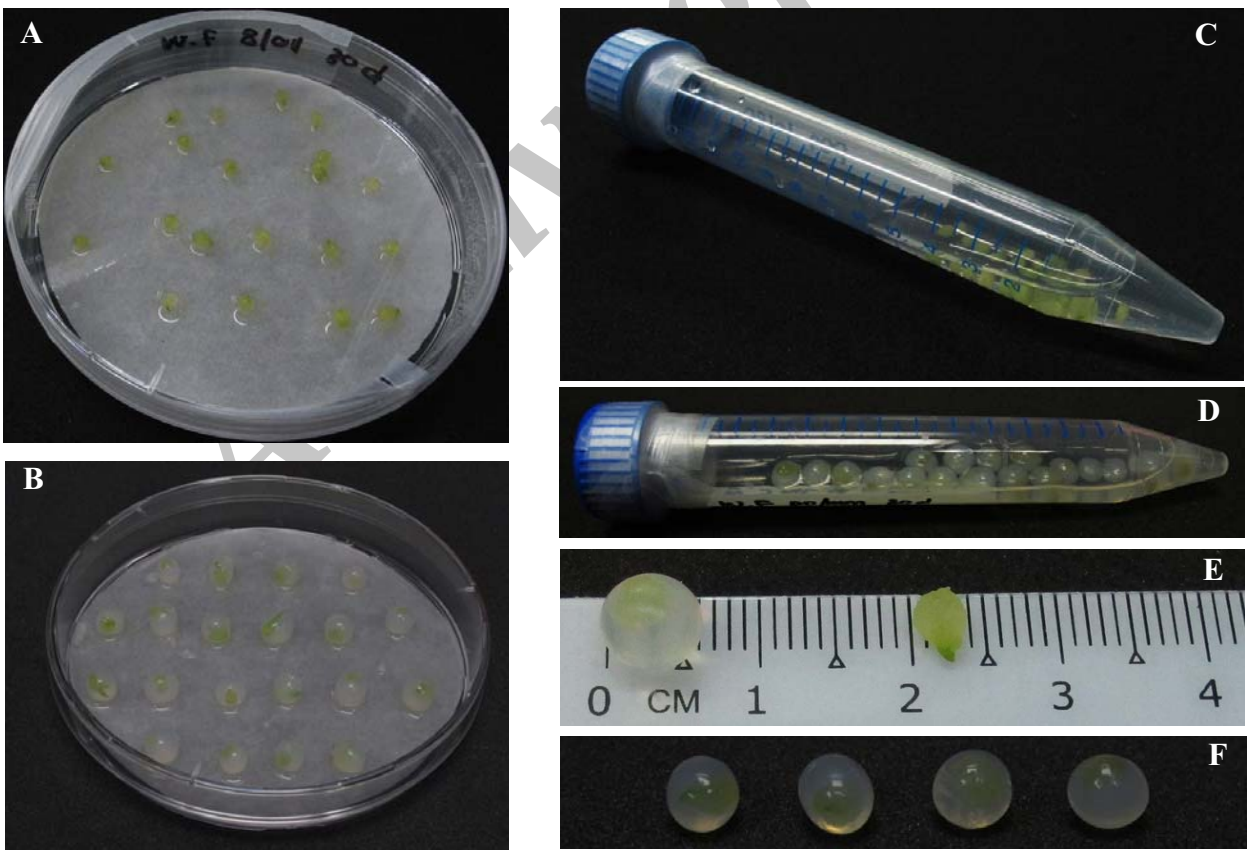

Fig. 1. Encapsulated and non-encapsulated *Dendrobium* White Fairy PLBs
12-week old *Dendrobium* White Fairy orchid PLBs (about 3 to 4 mm in diameter) used for the study. Non-encapsulated PLBs (A) and encapsulated PLBs (B) stored in Petri dish with 2mL of sterile water on a filter paper. Non-encapsulated PLBs (C) and encapsulated PLBs (D) stored in 15ml polypropylene tube (Falcon) containing 2ml of sterile water. Size range of the encapsulated PLBs (about 6mm) and non-encapsulated PLBs (about 4mm) (E).Calcium arginate beads containing PLBs for the experiments (F).

Table 1. Effect of storage time on the germination and conversion of encapsulated PLBs.

Storage Time (Day)	Percent of Germination		Percent of Conversion		Average number of plantlet with shoot of minimum 5mm length		Average number of plantlet with root of minimum 10mm length	
	Petri Dish	15ml PP Tube	Petri Dish	15ml PP Tube	Petri Dish	15ml PP Tube	Petri Dish	15ml PP Tube
0	95	95	35	35	4	4	2	2
30	100	97	18	24	2	4	1	0
60	90	80	8	17	1	1	0	1
90	100	95	28	48	5	7	3	4
120	98	92	38	60	4	10	2	6
150	87	88	33	43	7	9	3	4
180	0	77	0	44	0	4	0	3

Note: *Dendrobium* synthetic seeds stored in either Petri dish or 15 ml polypropylene (PP) tube at different storage times and storage containers were germinated on ½ MS media. Their percentage of germination, percentage of conversion, average shoot and root lengths were tabulated respectively after the 50-day cultivation period. Encapsulated PLBs at 0-day storage time point were used as controls.

4°C up to 120 days for a monopodial orchid species (Gantait *et al.,* 2012). Hence, in our study, 25°C was used as our storage temperature.

From table 1, a high germination percentage of more than 80% was observed for encapsulated synthetic seeds that were first stored in both Petri dish and screw capped PP tube and this germination percentage only began to decline after 150 days of storage. There is no major difference between the encapsulated seeds stored in Petri dish and PP tube at 25°C under dark conditions and this could be due to optimum storage conditions which allowed the uptake of nutrients from the half strength MS gel matrix to the seed to sustain its viability. The dark environment helped to delay PLB germination by keeping the PLBs at dormant stage. The maintenance of same temperature from storage of synthetic seeds to their cultivation at 25°C also imposed lesser physical harm to these PLBs as compared to storage at lower temperature due to the fluctuation in temperature and cold stress during the transfer. This observation was seen in a research done by Gantait *et al*. (2012). After 180 days of storage, synthetic seeds that were stored in the PP tubes were able to achieve 77% germination, while those stored in Petri dish had dried up and were unable to germinate at all. This could be because screw capped PP tubes are able to retain moisture content longer, and thus able to maintain their viability and prevent the synthetic seeds from drying up.

The conversion capability of the stored synthetic seeds into plantlets was also monitored after their respective storage time points for a period of 50 days. A similar trend was observed for the conversion percentage of *Dendrobium* White Fairy synthetic seeds as with the germination results shown in table 1.

Generally, synthetic seeds stored in PP tubes showed a slightly higher percent of conversion as compared to those stored in Petri dish and this difference in conversion rate between seeds stored in the two vessels generally increased with storage time. This could be because in addition to the PP tube's ability to retain moisture, the longer storage time also enabled the synthetic seeds to have ample time to adapt, grow and develop into mature PLBs with the available nutrients in the gel matrix, so that they are able to develop into complete plantlets faster due to their maturity stage. Synthetic seeds that were stored in PP tubes were also able to produce more plantlets with shoots and roots exceeding 5 mm and 10 mm respectively as compared to those that were stored in Petri dishes (Table1).

Based on the physical observation of the developed plantlets from synthetic seeds (Fig. 2).

Fig. 2. Phenotypes of synthetic seeds stored in Petri dish at 90-day time point at the end of 50 days of cultivation. (A) Synthetic seeds which were successfully converted into plantlets. (B) Some seeds grew slower and were still at the PLB stageat the end of cultivation. (C) About 15% to 20% of the plantlets appeared elongated. For those stored in screw capped polypropylene tubes, the elongated phenotype was also observed in about 15% to 20% of the plantlets, but only after 120 days of synthetic seed storage.

It was observed that when the encapsulated synthetic seeds were stored for 90 days or longer, the PLBs appeared yellow and unhealthy when they were first taken out from the dark, but when the PLBs were cultured onto ½ MS media, they gradually became green in colour and looked healthier (data not shown). About 15% to 20% of the plantlets which were stored for 90 days or longer also appeared elongated (Fig. 2C as compared to 2A and 2B). This could be due to gradual nutrient depletion when the encapsulated seeds were stored at longer time periods.

During the course of our experiments, an additional interesting observation was made as shown in Fig. 3.

Generally, longer storage period will lead to slower germination rate and lower germination capability and this was observed for the non-encapsulated seeds that were stored in Petri dishes for 30 and 60 days as compared to the 0-day control (Fig. 3B). The germination percentage also declined drastically from the 30-day storage to 60-day storage. The encapsulated PLBs that were stored in Petri dishes, on the other hand, were able to maintain a high germination percentage as expected (Fig. 3A).

In contrast, the PLBs stored in screw capped PP tubes maintained a high germination percentage regardless of its encapsulation status. Both encapsulated and non-encapsulated PLBs showed no reduction in germination rate as compared to the 0 day control for both the 30-day and 60-day storage time points (Fig. 3). They developed relatively well, just like a fresh seeds where there is no sign of delay in their germination ability. This could be due to the presence of water that was added to the tubes at the start of the experiment, which created a moist environment as well as the greater ability of screw capped PP tubes over Petri dishes to maintain a humid environment for the developing PLBs. This is crucial so as to prevent dehydration of the encapsulated seed or PLBs and in turn, preserve its viability (Gantait *et al.*, 2012).

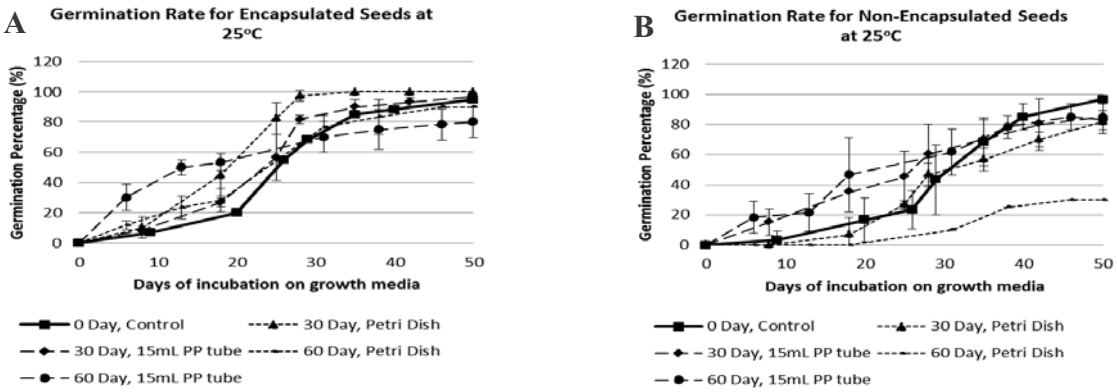

Fig. 3. Comparison of germination rate between encapsulated PLBs.
(A) and non-encapsulated PLBs, (B) stored in either Petri dish or 15ml PP tube for 0, 30, and 60 days. Cultivation conditions: 25±1°C, 16-hour photoperiod, 50-day cultivation period.

CONCLUSION

The synthetic seed technology can prove to be an effective method to preserve Orchid seeds for large scale propagation of these beautiful ornamental species or for import and export purposes based on other previous studies and our research findings. Our studies showed that *Dendrobium* White Fairy can be stored at 25oC in the dark as encapsulated beads in both Petri dishes and screw capped PP tubes, in which the later has shown to be more favourable. This study has also proved that non-encapsulated protocorms can be stored directly in screw capped PP tubes without the need to encapsulate, with the ability to maintain a high germination percentage of about 80% after 60 days of storage at 25oC in the dark. With these findings, we have shown a more simplified way of storing and propagating *Dendrobium* White Fairy PLBs. However, more research would be required to study and optimize the storage procedure for this orchid species.

ACKNOWLEDGEMENTS

The authors would like to thank Professor Chia Tet Fatt for his valuable technical advice, Mrs Khoo-Woon Mui Hwang, the Singapore Botanic Gardens, for providing technical training. The study is financially supported by the Ministry of Education, Singapore (Grant No. MOE2009-IF-1-012).

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