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Effect of Encapsulation Parameters and Storage Conditions on Synthetic Seeds of *Vanda bensonii* Batemen

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Key words: Synthetic seed technology, Encapsulation, Sodium alginate, Calcium chloride, Protocorm-like bodies (PLBs), Regeneration potential

Abstract

The present study investigates the medium-term storage of regenerated plants of *Vanda bensonii* Batemen using synthetic seed technology, wherein protocorm-like bodies (PLBs) were encapsulated in sodium alginate and complexed with calcium chloride to produce bead-like propagules capable of regenerating into complete plantlets. Bead morphology was influenced by the concentrations of sodium alginate and calcium chloride, with 3% (w/v) sodium alginate and 100 mM $CaCl_2$ producing mostly globular, tail-free beads that ensured good encapsulation integrity and handling efficiency. Increasing $CaCl_2$ concentration to 200 mM led to uneven cross-linking and distorted bead shapes, indicating that 100 mM $CaCl_2$ offers near-optimal gelation conditions. The regeneration potential of encapsulated PLBs was assessed after storage at different temperatures and durations. At 4°C, the PLBs exhibited high viability, with regeneration rates of 95.16% at 15 days, 92.73% at 30 days, and 85.71% at 45 days, followed by a gradual decline over longer storage periods. Although a slight recovery was observed at 75 days (90.16%), viability significantly decreased after six months, reaching 53.33% at 270 days. Conversely, storage at 16°C resulted in rapid deterioration, with regeneration falling from 36.36% at 15 days to complete loss beyond 45 days. These results demonstrate that low-temperature storage (4°C) is markedly superior for maintaining the viability and regeneration potential of synthetic seeds by slowing metabolic degradation.

Introduction

Orchids (family Orchidaceae) constitute one of the largest and most diverse families of flowering plants, comprising more than 25,000 species distributed worldwide (Dressler 1993). Many members of this family, including *Vanda* species, are of high ornamental and commercial importance but face increasing threats from habitat loss, over collection, and limited natural regeneration (Pant 2013). *Vanda bensonii* Batemen is a rare and attractive

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epiphytic orchid valued for its horticultural potential and ecological significance; however, its propagation remains constrained by the species' slow growth rate and dependency on specific symbiotic associations for seed germination. Hence, developing efficient *in vitro* propagation and storage techniques is critical for its conservation and sustainable utilization.

Synthetic seed technology, also known as artificial seed production, has emerged as a promising strategy for large-scale propagation and medium-term conservation of elite and endangered orchid germplasm (Rao et al. 1998). This method involves encapsulating propagules such as protocorm-like bodies (PLBs), shoot tips, or somatic embryos in a gel matrix (typically sodium alginate) complexed with calcium chloride, forming bead-like structures capable of regenerating into complete plantlets under suitable conditions (Sharma et al. 2013). PLBs are particularly advantageous as encapsulated materials due to their high morphogenic competence and genetic stability (Arditti and Ernst 1993). Previous studies on related taxa—such as *Cymbidium finlaysonianum* (Thammasiri and Soamkul 2007) and *V. tessellata* (Manokari et al. 2021)—have demonstrated that low-temperature storage of encapsulated PLBs can effectively maintain viability and regeneration potential for several months.

Despite such progress, information regarding synthetic seed production and storage in *V. bensonii* remains limited. The present study focuses on optimizing encapsulation parameters, specifically sodium alginate and calcium chloride concentrations, and assessing the effects of storage temperature and duration on the regeneration potential of encapsulated PLBs. The results aim to establish a reliable synthetic seed protocol for *V. bensonii*, thereby supporting both commercial propagation and *ex situ* germplasm conservation efforts.

Materials and Methods

Healthy plants of *V. bensonii* established in the departmental garden of the Department of Botany, Visva-Bharati, were selected for the experiment. Flowers were manually pollinated and subsequently left undisturbed until successful capsule development. The pollinated flowers produced mature capsules after approximately five months. At maturity, the capsules turned golden brown and contained numerous minute, powdery seeds.

Twelve-week-old, *in vitro* generated (PLBs), measuring approximately 3-4 mm in diameter, were used for synthetic seed production. Encapsulation was performed aseptically following the method of Lambardi et al. (2006). Individual PLBs were suspended in a 3% (v/v) sodium alginate solution and dispensed as single drops using a micropipette tip with a 7 mm opening into a 100 mM calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) solution, as described by Gantait et al. (2012). The droplets were maintained in the $CaCl_2$ solution for 30 min with gentle stirring to prevent aggregation. The resulting calcium alginate beads were then carefully removed, rinsed three times with sterile distilled water, and gently blotted dry using sterilized filter paper.

For storage experiments, the encapsulated PLBs were evaluated to determine the effects of different storage durations and two temperature regimes on germination and conversion efficiency. Non-encapsulated PLBs served as controls. Storage periods included 15, 30, 45, 60, 90, 180, and 270 days, with three replicates of 40 PLBs per time point. For each replicate, the synthetic seeds were split into two groups and stored at either 4°C or 16°C, with each storage container containing blotting paper dipped in 5 ml of sterile water to maintain humidity. Control PLBs were stored under the same conditions and assessed at 0, 30, and 60 days. All samples, encapsulated and non-encapsulated, were kept in darkness at their respective temperatures until the completion of the designated storage period.

After exposure to different storage conditions and storage durations, the artificial seeds were retrieved from the Petri plates and assessed for their regeneration potential. The stored calcium alginate beads were aseptically transferred onto half-strength ½MS medium supplemented with 2-isopentenyl adenine (2iP), 2mg/l and Na_2EDTA, 37.25 mg/l. Cultures were incubated under controlled growth room conditions, and regeneration responses were recorded in terms of bead swelling, protocorm re-greening, shoot emergence, and complete plantlet formation. Regeneration frequency and growth performance were evaluated at regular intervals following inoculation.

Genomic DNA was isolated from plant tissues using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. Approximately 100 mg of fresh plant tissue was ground into a fine powder in liquid nitrogen using a sterile mortar and pestle and transferred into a 1.5 ml microcentrifuge tube. To this, 400 µl of Buffer AP1 and 4 µl of RNase A were added, mixed thoroughly by vortexing, and incubated at 65°C for 10 min with intermittent mixing. After lysis, 130 µl of Buffer AP2 was added, and the mixture was incubated on ice for 5 min before centrifugation at 20,000 × g for 5 min to pellet debris. The supernatant was transferred to a QIA shredder spin column and centrifuged again at 20,000 × g for 2 min. The flow-through was mixed with 1.5 volumes of Buffer AW1 and passed through a DNeasy mini spin column in two steps, each followed by centrifugation at 6,000 × g for 1 min. The column was washed twice with 500 µl of Buffer AW2, first centrifuged at 6,000 × g for 1 min and then at 20,000 × g for 2 min to remove residual ethanol. DNA was eluted by adding 50-100 µl of Buffer AE, incubating at room temperature for 5 min, and centrifuging at 6,000 × g for 1 min. The purified DNA was stored at -20°C for long-term use or at 4°C for short-term storage.

Inter-simple sequence repeat (ISSR) analysis was performed using a 15 µl PCR reaction mixture containing 1.0 µl of plant genomic DNA (10-100 ng), 0.6 µl of a single ISSR primer (10 µM), 0.3 µl of dNTP mix (10 mM each), 3.0 µl of 10× PCR buffer, 0.6 µl $MgCl_2$, 0.3 µl of Taq DNA polymerase (5 U/µl), and 9.2 µl of nuclease-free water to make up the final reaction volume. PCR amplification was carried out in a thermal cycler with an initial denaturation at 94°C for 3 min, followed by 44 cycles of denaturation at 94°C for 30 sec, annealing at 52°C (depending on primer melting temperature) for 30 sec, and extension at 72°C for 30-60 sec. A final extension was performed at 72°C for 5-10 min, and the reactions were held at 4°C until further analysis. This ISSR primer used in this study is presented in Table 1.

Table 1. ISSR primer used for assessment of genetic stability.

ISSR Primer	Sequences	Annealing Temperature (°C)
UBC 836	5'- AGAGAGAGAAGAAGAGYG -3'	52

The PCR amplicons were separated on a 1.7% (w/v) agarose gel prepared in 1× TAE buffer and run at 90 V. A 1 kb DNA ladder (GCC BIOTECH) served as the molecular size standard. Gels were stained with Ethidium bromide (10 mg/ml) and visualized using a Gel Documentation System (Chemi Doc, Bio-Rad).

All experiments were conducted in triplicate, and the results are expressed as mean ± standard error (SE). Statistical analyses were performed using Graph Pad Prism software. Differences among treatment groups were evaluated using one-way analysis of variance (ANOVA), followed by appropriate post-hoc multiple comparison tests. A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Bead morphology during encapsulation of *V. bensonii* PLB was strongly influenced by both sodium alginate and calcium chloride concentrations (Table 2). Beads prepared with 2% sodium alginate, irrespective of the $CaCl_2$ concentration (100-300 mM), were extremely soft, fragile, and mechanically unstable, making them unsuitable for handling and storage. Increasing the sodium alginate concentration to 3% improved bead firmness and structural integrity; however, at 100 mM $CaCl_2$, the beads appeared nearly spherical with slight tailing, whereas at 200 mM, they exhibited more pronounced tapering at the ends due to uneven cross-linking at higher calcium levels. Further increase to 300 mM $CaCl_2$ produced overly pliable beads lacking sufficient rigidity. At 4% sodium alginate, the beads became excessively viscous and deformed, resulting in irregular or asymmetrical shapes at 100-200 mM, and equidimensional but slightly flattened forms at 300 mM. Overall, 3% sodium alginate in combination with 100 mM $CaCl_2$ yielded the most desirable bead morphology—uniform, nearly spherical, and sufficiently firm for handling—indicating this combination as optimal for synthetic seed formation in *V. bensonii*.

Table 2. Characteristics of synthetic beads in various concentrations of sodium alginate as well as calcium chloride

Sodium alginate (%; w/v)	Calcium chloride concentration (mM)	Bead characteristics
2	100 mM	Extremely soft and fragile
2	200 mM	Poorly solidified; mechanically unstable
2	300 mM	Poorly solidified; mechanically unstable
3	100 mM	Nearly spherical with slight tailing
3	200 mM	Irregular with pronounced tapering ends
3	300 mM	Irregular with pronounced tapering ends
4	100 mM	Non-uniform and asymmetrical
4	200 mM	Non-uniform and asymmetrical
4	300 mM	Non-uniform and asymmetrical

Various stages of PLB production, encapsulation and regeneration from encapsulated PLBs have been presented in Fig. 1.

The regeneration behaviour of encapsulated PLBs of *V. bensonii* was found to be strongly influenced by both storage temperature and duration. At 4°C, the encapsulated PLBs maintained high regeneration efficiency during the initial storage periods, confirming that low-temperature conditions are favourable for preserving viability (Table 3). After 15 days, regeneration reached its peak at 95.16% (59 out of 62 seeds germinated), with only a slight decrease observed at 30 days (92.73%) and 45 days (85.71%). By 60 days, regeneration dropped to 79.59%, indicating the onset of gradual viability loss. Interestingly, a moderate increase to 90.16% at 75 days suggested some variability in storage response, possibly due to differences in the physiological status or vigour of the PLBs at the time of encapsulation. However, beyond 90 days, regeneration declined progressively to 83.64% at 90 days, 72.22% at 180 days, and the lowest at 53.33% after 270 days, demonstrating that prolonged storage beyond six months leads to a significant reduction in germination potential.

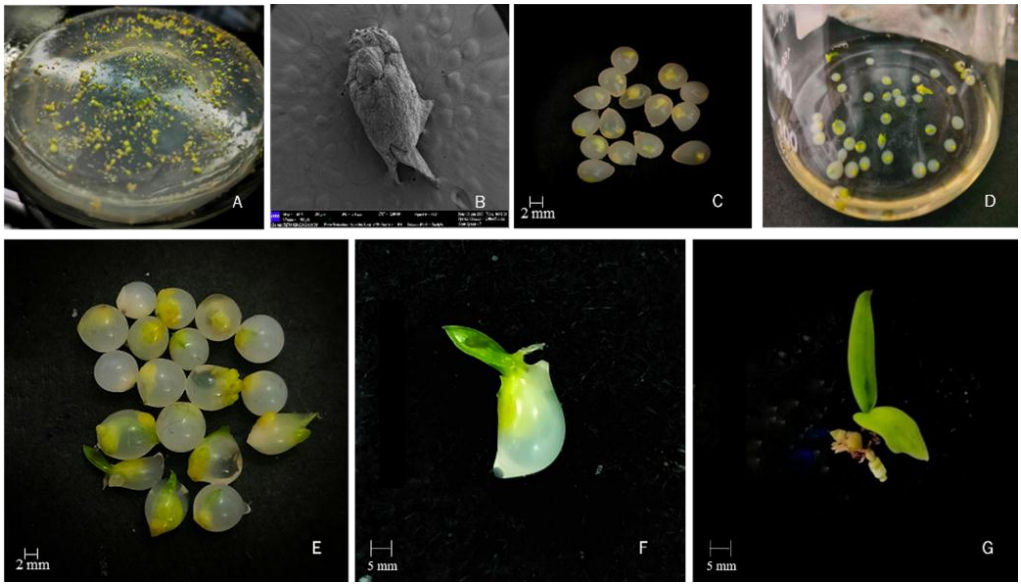

Fig. 1. (A) PLBs produced from seed suspension culture, (B) SEM of a single PLB, (C) encapsulated PLB, (D-F) regeneration of plantlets from encapsulated PLBs of *V. bensonii* after storage at 4°C for 60 days, and (G) a fully grown *V. bensonii* plant obtained from an encapsulated PLB, stored at 4°C for 60 days.

In contrast, PLBs stored at 16°C exhibited a sharp decline in viability even at early time intervals. Regeneration was limited to 36.36% after 15 days and dropped to 23.91% at 30 days and 11.11% at 45 days, after which no germination was recorded. This rapid deterioration suggests that moderate temperatures accelerate metabolic activity, resulting in higher respiration rates and depletion of stored reserves.

Table 3. Regeneration of plantlets from encapsulated PLBs of *V. bensonii* after storage at different temperatures for different durations.

Temperature	Storage duration (days)	No. of seeds prepared	No. of germinated PLBs	Regeneration potential of encapsulated PLBs (%)
4°C	15	62	59	95.16 ± 0.21**
	30	55	51	92.73 ± 0.38**
	45	49	42	85.71 ± 0.51ns
	60	49	39	79.59 ± 0.27ns
	75	61	55	90.16 ± 0.26ns
	90	55	46	83.64 ± 0.41ns
	180	54	39	72.22 ± 0.39*
	270	45	24	53.33 ± 0.45ns
16°C	15	44	16	36.36 ± 0.74ns
	30	46	11	23.91 ± 0.24ns
	45	45	5	11.11 ± 0.42ns
	60	55	-	-
	75	52	-	-
	90	38	-	-
	180	39	-	-
	270	46	-	-

Values are expressed as mean ± standard error (SE). Asterisks indicate statistically significant differences (Tukey's test, $p < 0.05$). Statistical analysis was performed using Graph Pad Prism version 9.0

Genetic stability of the synthetic seed-derived plants of *V. bensonii* was assessed using Inter-Simple Sequence Repeat (ISSR) marker-UBC 836 (Fig. 2). Young leaf tissues were collected from the mother plant and nine randomly selected regenerants. ISSR marker-UBC 836 analysis revealed a uniform, monomorphic banding profile across the mother plant and nine randomly selected regenerants. The absence of polymorphic bands indicates the lack of detectable somaclonal variation, suggesting that the micropropagation protocol employed maintains genetic fidelity.

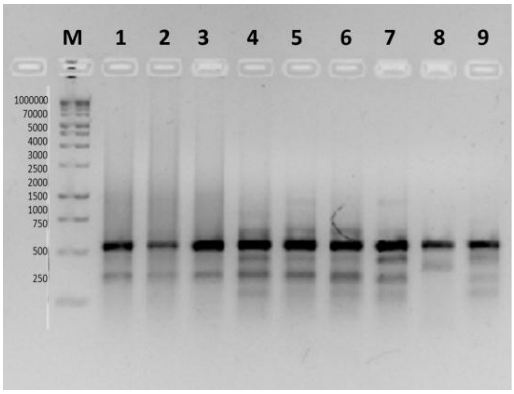

Fig. 2. ISSR profile of *Vanda bensonii* generated using primer UBC 835. Lane M: 1 kb DNA ladder; Lane 1: mother plant; Lanes 2-9: synthetic seed (stored at 4°C for 60 days) derived regenerants. All lanes exhibit a monomorphic banding pattern, indicating genetic uniformity among regenerants and confirming clonal stability.

The morphology of synthetic beads during encapsulation of *V. bensonii* PLBs was strongly influenced by sodium alginate and calcium chloride concentrations. Bead shape is a critical factor in synthetic seed technology, as globular, tail-free beads ensure structural integrity, ease of handling, uniform gelation, and optimal germination (Kole and Giri 2015). The combination of 3% sodium alginate with 100 mM $CaCl_2$ produced predominantly globular beads with minor tail formation, indicating near-optimal gelation. This suggests that at this concentration, cross-linking between alginate chains and calcium ions occurs at a controlled rate, forming stable beads without significant distortion. Increasing calcium chloride concentration to 200 mM resulted in more pronounced tail formation, likely due to rapid and uneven cross-linking. Lower sodium alginate concentrations (2%) produced beads too soft to handle regardless of calcium levels, whereas higher alginate concentrations (4%) in combination with any calcium concentration led to deformed or isodiametric beads, reflecting overly dense or rigid matrices. Collectively, these observations indicate that 3% sodium alginate with 100 mM calcium chloride, coupled with optimized gelation time, is most suitable for generating structurally robust synthetic seeds.

Regeneration potential of encapsulated PLBs was strongly dependent on storage temperature and duration. At 4°C, PLBs maintained high viability for up to 90 days, with regeneration percentages ranging from 95.16% at 15 days to 83.64% at 90 days. Even after 180 days, over 70% of PLBs regenerated, demonstrating that low-temperature storage effectively slows metabolic activity, minimizes oxidative damage, and preserves embryogenic potential. A slight increase at 75 days (90.16%) may reflect variability in PLB physiological status or bead quality. Extended storage beyond nine months led to significant loss of viability, with only 53.33% regeneration at 270 days, highlighting limitations of long-term storage even under optimal low-temperature conditions. In contrast, storage at 16°C resulted in a rapid decline, with regeneration falling from 36.36% at 15 days to complete loss after 45 days, suggesting that moderate temperatures accelerate metabolic degradation and depletion of stored reserves. These results align with previous reports emphasizing that low-temperature storage is essential for maintaining the viability of encapsulated orchid propagules (Engelmann 2004, Rout et al. 2012).

Genetic stability is a crucial consideration in orchid micropropagation, as somaclonal variation can compromise uniformity and horticultural value. ISSR marker analysis of *V. bensonii* regenerants in this study revealed monomorphic banding across all loci, confirming the absence of detectable somaclonal variation. This indicates that PLB-mediated regeneration maintains clonal fidelity, consistent with earlier studies showing that direct organogenesis or PLB pathways minimize genetic variation compared to callus-based systems (Martin and Madassery 2006, Lakshmanan et al. 2007).

Overall, these findings underscore two critical aspects of synthetic seed technology in *V. bensonii*: (i) careful optimization of sodium alginate and calcium chloride concentrations is necessary to produce structurally stable, nearly spherical beads, and

(ii) low-temperature storage at 4°C is essential for sustaining high regeneration potential over medium-term storage. This combination offers a reliable strategy for propagation, conservation, and distribution of valuable orchid germplasm.

The present study demonstrates that synthetic seed technology is an effective approach for the medium-term storage and propagation of *V. bensonii*. Encapsulation of PLBs in 3% sodium alginate with 100 mM calcium chloride produced beads with optimal morphology, providing mechanical protection and handling stability. Storage at 4°C preserved high regeneration potential for up to three months, while storage at 16°C led to rapid viability loss. These results highlight the importance of optimizing both encapsulation parameters and storage conditions to maintain the viability and embryogenic potential of synthetic seeds.

Future research can focus on further improving the efficiency of this technology by incorporating growth regulators, osmotic agents, or nutrients within the alginate matrix to enhance germination and plantlet establishment. Additionally, exploring cryopreservation, longer-term storage, and ex vitro conversion under greenhouse conditions can expand the practical application of synthetic seeds for commercial propagation and conservation of rare and threatened orchids. Integration of this approach with molecular markers could also ensure genetic fidelity, supporting sustainable production and germplasm preservation strategies for *V. bensonii* and related ornamental species. Overall, synthetic seed technology provides a reliable and efficient system for the medium-term conservation and propagation of *V. bensonii*, contributing to both its commercial cultivation and long-term germplasm preservation. This technique provides an efficient approach for large-scale propagation, germplasm conservation, and safe exchange of viable materials.

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