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ACKNOWLEDGEMENT. We thank the Board of Research in Nuclear Sciences (BRNS), Department of Atomic Energy for providing financial assistance.

Received 20 July 2009; revised accepted 5 February 2010

Artificial seed production from encapsulated PLBs regenerated from leaf base of *Vanda coerulea* Grifft. ex. Lindl. – an endangered orchid

Debojit Kumar Sarmah[1,*], M. Borthakur[1] and P. K. Borua[2]

[1]North East Institute of Science and Technology, Jorhat 785 006, India
[2]Department of Life Sciences, Dibrugarh University, Dibrugarh 786 004, India

Artificial seeds were produced from encapsulated protocorm-like bodies (PLBs) obtained from six-month-old axenic leaf explants of *Vanda coerulea* Griff. ex. Lindl. The percentage of germination of encapsulated PLBs was influenced by the concentrations of sodium alginate and calcium chloride ($CaCl_2 \cdot 2H_2O$) used. It was found that among the different concentrations tested, 3% sodium alginate and exposure to 100 mM $CaCl_2 \cdot 2H_2O$ solution for 30 min produced firm, clear, round and uniform optimal beads which were suitable for handling. It was also observed that PLBs obtained from optimization of encapsulation matrix showed the highest percentage of germination (94.9%) when beads were innoculated immediately after formation. Encapsulated PLBs stored at 4°C retain their viability up to 100 days. The findings suggest that the encapsulation method for PLB obtained from leaf explants of *V. coerulea* can be useful as an alternative tool for conservation of this endangered species.

Keywords: Encapsulation, ecorehabilitation, *in vitro*, protocorm-like bodies.

THE production of synthetic seeds in orchids is useful since orchids produce tiny and non-endospermic seeds.

*For correspondence. (e-mail: sarmah_debojit@rediffmail.com)

Review of the literature suggested that so far no reports have been published on the production of artificial seeds from encapsulated protocorm-like bodies (PLBs) derived from leaf explants of *Vanda coerulea*. The main aim of this work was to determine the optimal concentration of encapsulation matrix (sodium alginate solution and calcium chloride ($CaCl_2 \cdot 2H_2O$) solution) and optimal duration of exposure to hardening solution and the percentage of germination in order to produce ideal beads. At the same time, optimal duration of storage for germination of beads was also determined.

Leaf explants of *V. coerulea* were obtained from *in vitro* plantlets (six months old). Intact leaf was cultured on Ichihashi and Yamashita (IY)[1] basal medium supplemented with 20 g l^{-1} sucrose and 0.8% agar with different concentrations of thidiazuron (TDZ) and naphthaleneacetic acid (NAA). The pH of the medium was adjusted to 5.6 prior to autoclaving at 121°C for 21 min. All cultures were incubated under light intensity of 2000 lux 16/8 h light/dark photoperiod at 25 ± 2°C temperature and 70–80% relative humidity. PLBs of approximately 3.0 mm in diameter grew directly on leaf explants after 6 weeks in culture (Figure 1 *a*). They were isolated from the surface of the leaf explants (Figure 1 *b*) and encapsulated.

For encapsulation, various concentrations of sodium alginate solution (1–4% w/v) were prepared in IY basal medium solution (pH 5.6) with 20 g l^{-1} sucrose.

Observations were made after beads formation in $CaCl_2 \cdot 2H_2O$. It was found that encapsulated PLBs showed different degrees of success based on bead formation (Table 1). The beads which were formed at higher concentration of sodium alginate solution (4%) and dipped in 100 mM $CaCl_2 \cdot 2H_2O$ solution were rigid, firm, clear and isodiametric and suitable for handling (Figure 1 *c*). Beads thus formed were hard and dropping of alginate solution from pipette to $CaCl_2 \cdot 2H_2O$ solution was very slow. On the other hand, beads that were formed in 4% sodium alginate solution and dipped in 50/75 mM $CaCl_2 \cdot 2H_2O$ solution were of uniform size, isodiametric, solid and quite rigid. These beads had a short tail on the surface (Figure 1 *d*). The beads, which were formed by using 3% sodium alginate solution and 100 mM $CaCl_2$ solution as a complexation process were of uniform size, isodiametric, clear, solid and were ideal for the present study (Figure 1 *e*). On the other hand, beads formed in 3% sodium alginate and hardened with 50/75 mM $CaCl_2 \cdot 2H_2O$ solution were of uniform size, solid with short tail on the surface (Figure 1 *f*). Beads with 2% sodium alginate and hardened in 100 mM $CaCl_2$ solution had solid texture with clusters (Figure 1 *g*) and those hardened in 50/75 mM $CaCl_2 \cdot 2H_2O$ solution gave malformed beads, which were too soft to handle (Figure 1 *h*). At lower concentration of sodium alginate (1%) and higher concentration of $CaCl_2 \cdot 2H_2O$ 75–100 mM bead formation was very poor, i.e. PLBs did not get coated. No beads or capsules were produced in 50 mM $CaCl_2$ solution.

Further experiments were conducted to evaluate the germination of encapsulated PLBs, the capability of the PLBs to break the gel coat and to continue normal growth resulting in the emergence of shoots and roots. The beads obtained from different concentrations of alginate solution and $CaCl_2$ solution showed variable percentage of germination and also required different time intervals (Table 2). The beads that were formed in 3% sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$ solution showed 94.9% germination in IY basal medium within one week. PLBs encapsulated in 3% alginate matrix in 75 mM $CaCl_2 \cdot 2H_2O$ exhibited 56% germination after 8 days. The germination percentage of PLBs, encapsulated with 4% alginate and hardened in 100 mM $CaCl_2 \cdot 2H_2O$ resulted in minimum germination (23%) after 14 days, whereas PLBs encapsulated in 4% sodium alginate with 75 mM gave 36% germination after 9 days.

Based on the result obtained, it was obvious that the concentration of sodium alginate and complexing agent ($CaCl_2 \cdot 2H_2O$) influenced the frequency of bead to plant conversion. At higher concentration of sodium alginate (4%), beads formed were harder and suppressed the emergence of shoot apices. Tejavathi[2] worked on encapsulation of *in vitro* shoot buds and somatic embryos of *Agave vera-cruz* Mill and reported that at higher concentration of sodium alginate, beads were hard, which suppressed the emergence of the shoot/root or both thereby decreasing the conversion frequency. The results were in conformity with the present study. At lower concentration, sodium alginate (2%) was not suitable for encapsulation because the beads were formed without a definite shape, resulting in reduction of germination. In the present study, it was observed that 3% sodium alginate coated in 100 mM $CaCl_2 \cdot 2H_2O$ solution for encapsulation was the optimal concentration to produce artificial seeds from encapsulated PLBs.

The results further indicated that the frequency of bead to plant conversion also depended on the duration of exposure to $CaCl_2 \cdot 2H_2O$ for the hardening process. Exposure time of 30 min in complexing gel resulted in the best beads and is referred to as optimal period (Figure 2). On the other hand, the percentage of germination for encapsulated PLBs was minimum (40%), when the hardening period was short, i.e. 10 min. Beyond 30 min of hardening was detrimental for germination.

Sodium alginate (3%) and $CaCl_2 \cdot 2H_2O$ (100 mM) solutions were found to be the optimal concentration for ideal bead formation. The beads were stored for different time intervals at 4°C to conduct germination experiments. The encapsulated PLBs (without storage), when germinated directly on medium showed conversion frequency of 94.9% after one week. The synthetic seeds stored at 4°C remained viable and germinated up to 100 days. However, poor germination of 6.5% was observed after completion of 100 days of storage. Gradual declination of germination of synthetic seeds was observed when

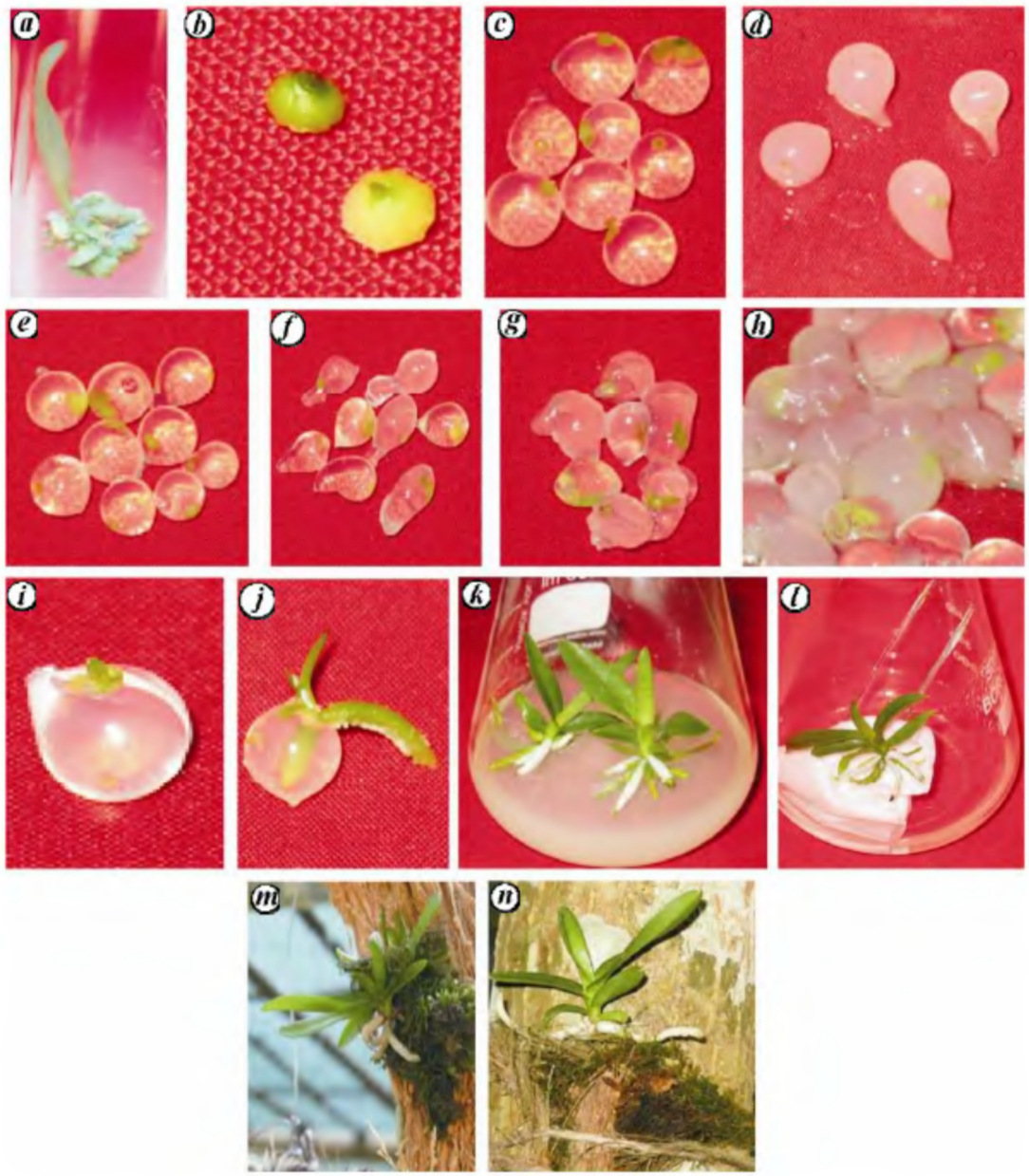

Figure 1. ***a***, PLB formation in cultures of leaf base of *Vanda coerulea*. ***b***, Isolated PLB from leaf base (enlarged). Encapsulated seeds in ***c***, 4% and 100 mM; ***d***, 4% and 75/50 mM; ***e***, 3% and 100 mM; ***f***, 3% and 75/50 mM; ***g***, 2% and 100 mM; ***h***, 2% and 75/50 mM sodium alginate and $CaCl_2 \cdot 2H_2O$ solution respectively. ***i***, Germinated encapsulated beads. ***j***, Appearance of shoot and roots. ***k***, Complete plant developed from encapsulated beads. ***l***, Hardening of plants. ***m***, Hardened plants with primary binding substratum (coco pith and moss). ***n***, Plant established in tree trunk.

storage time was increased. After 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 days storage at 4°C, the conversion frequencies were 78%, 65.5%, 52%, 41%, 25%, 15.3%, 13.5%, 10%, 8% and 6.5% respectively (Figure 3). The time required for germination of synthetic seeds was found to be different, when seeds were stored at 4°C for different time intervals. In seeds cultured after 10 days storage, germination response was observed within 12–15 days. Those cultured after 20 days storage took longer time for germination. The maximum time period (20 days) for germination of synthetic seeds was observed when synthetic seeds were stored for 80–100 days.

The germinated encapsulated beads (Figure 1 *i*) showed varied responses of shoot and root initiation. Some beads developed both shoot and root simultaneously after 10–12 days from the date of appearance of vegetative peak at the surface of synthetic beads (Figure 1 *j*). On subculture in fresh medium, the plantlets developed both well-grown

Table 1. Effect of different concentrations of sodium alginate and $CaCl_2 \cdot 2H_2O$ on formation of encapsulated beads

Concentration of sodium alginate (%)	Concentration of $CaCl_2 \cdot 2H_2O$ (mM)	Nature of bead formation	Remarks
1	50	+	Fail to coat protocorm-like bodies
1	75	++	Too soft and very fragile
1	100	++	Poor bead formation
2	50	+	Malformed beads, very fragile and soft to handle
2	75	++	Solid texture and formed clusters
2	100	++	Rigid, solid texture and form cluster
3	50	+++	Uniform size, solid and isodiametric
3	75	+++	Uniform size, solid and sort tail at the surface
3	100	+++++	Clear, firm, round and uniform size
4	50	++++	Uniform size and isodiametric
4	75	++++	Uniform size, isodiametric and quite rigid
4	100	+++++	Rigid, firm, clear and isodiametric

+, Poor quality; ++, Poor quality; +++, Slight better; ++++, Good but solid; +++++, Best quality.

Table 2. Effect of different concentrations of sodium alginate (%) and $CaCl_2 \cdot 2H_2O$ (mM) on germination percentage of encapsulated PLBs on Ichihashi and Yamashita basal medium without storage

Sodium alginate (%)	$CaCl_2 \cdot 2H_2O$ (mm)	Days for germination	Percentage of germination
3	75	8	56.5 ± 0.45
3	100	7	94.9 ± 0.48
4	75	9	36.5 ± 0.45
4	100	14	23.5 ± 0.71

±, Standard error.

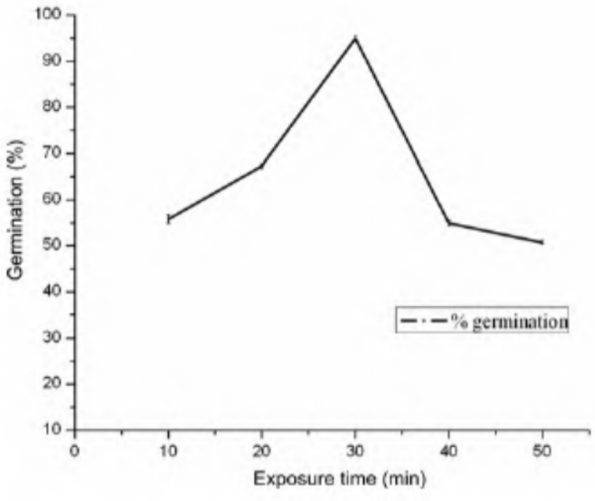

Figure 2. Germination percentage of encapsulated PLBs in relation to different exposure time to $CaCl_2 \cdot 2H_2O$ solution. (After 10 weeks of culture.)

shoots and roots (Figure 1 *k*). Well-developed plantlets regenerated from encapsulated PLBs were successfully hardened in the same cultural condition for 15 days (Figure 1 *l*). Hardened plants were binded with coco pith and moss, and acclimatized in the greenhouse for a eco-rehabilitation month (Figure 1 *m*). During this time, adequate watering was done once a week. The plants were then established on different tree trunks for their ultimate ecorehabilitation in natural conditions (Figure 1 *n*).

The regeneration of orchid plants through encapsulation of tissue culture derived propagules in a nutrient gel has initiated a new line of research on synthetic seeds. The success of retrieving complete plantlets *in vitro* from encapsulated axillary buds/shoot tips/somatic embryos, though reported in several herbaceous species, there are only a few reports on the propagation of orchids using synthetic seeds[3–5]. Soneji[6] reported that a concentration of 3% sodium alginate was most effective for shoot encapsulation in *Ananas cosmosus*. Daud[7] also reported 3% sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$ was found to be effective for encapsulation of micro shoots of *Saintpaulia ionantha*. Awal[8] reported that encapsulation of micro shoots of *Begonia* × *Hiemalis* Fotch in 3% sodium alginate gave 90.48% germination. Nayak[5] reported that 4% sodium alginate and 75 mM $CaCl_2 \cdot 2H_2O$ were most suitable for formation of clear beads. However, these beads germinated (beads to plant conversion) within 10–20 days

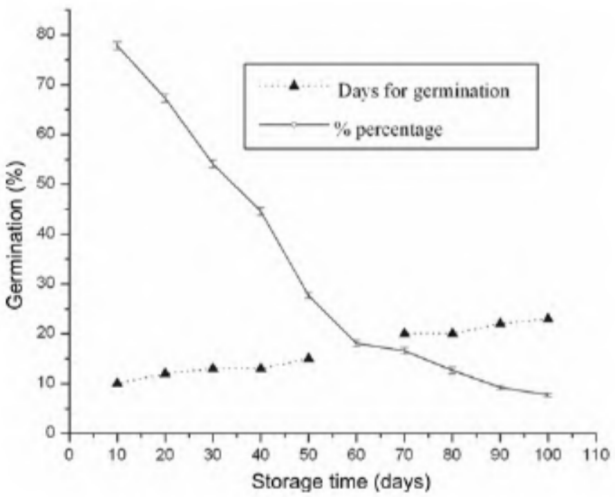

Figure 3. *In vitro* germination percentage and time requirement for germination of encapsulated PLBs (stored at 4°C) in relation to different period of storage (days).

and germination frequency was lower compared to the present study. Sharma[3] reported that encapsulated PLBs of *Dendrobium wardianum* showed 100% conversion frequency after 45 days of culture which are similar with the present findings. They also reported that synthetic seeds stored at 4°C could reduce the germination percentage, which was observed in the present study. Higher germination percentage in case of synthetic seeds (without storage) could be due to the matrix, which not only facilitates regular nutrient supply but also protects delicate tissue from any mechanical injury during handling and from desiccation. According to Redenbaugh[9], the beads can potentially serve as a reservoir of nutrients that may aid survival and speed up growth. The alginate matrix containing nutrients reduced the viscosity and ability of the gel to form solid beads. Germination frequency of synthetic seeds also depended on the exposure time in complexing gel. In the present study, 100% of bead to plant conversion was observed in 30 min exposure to $CaCl_2{\cdot}2H_2O$ solution, with 75–78% germination in more than 30 min exposure. Hardness in capsules causing anaerobic environment inside the capsules may inhibit PLB respiration. Hardness or rigidity of the beads mainly depends on the number of Na^+ ions exchanged with Ca^- ions. At the same time, internal factors related to developmental stage of PLBs could also be one of the important limiting factors affecting germination.

Thus, the present study showed that the germination percentage of encapsulated PLBs was affected by the complexing gel and duration of exposure to $CaCl_2{\cdot}2H_2O$ solution. Using 3% sodium alginate solution and maintaining in 100 mM $CaCl_2{\cdot}2H_2O$ solution for 30 min gave the optimal concentration and highest percentage of germination (94.9%). The result so obtained from encapsulation of PLBs can be used as a potential method to solve the problems of propagation for monopodial orchids like *V. coerulea* that have tiny seeds and lack of endosperm. The present study has demonstrated multiplication of PLBs from leaf tissue without damaging the mother plant. These PLBs were utilized for the production of uniform beads with high frequency of beads to plant conversion and easy handling which would be useful for mass propagation of endangered species like *V. coerulea.*

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ACKNOWLEDGEMENT. D.K.S. thanks the Director, NEIST, Jorhat for providing laboratory facilities and other support in conducting the investigation included in this paper as a part of Ph D work.

Received 25 February 2009; revised accepted 11 January 2010