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SYNTHETIC SEEDS AND *IN VITRO* PROPAGATION OF *CYMBIDIUM ALOIFOLIUM* (LINN.) SW.

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Abstract

Synthetic seeds (Synseeds/Artificial seeds) were successfully prepared in *Cymbidium aloifolium* by encapsulating PLBs (0.1-0.2 cm) in a hydrated gel capsule composed of calcium alginate (Sodium alginate; 3.5% and Calcium chloride; 75 mM) matrix in liquid Mitra *et al.*, 1976 (M) medium supplemented with IAA (1.0 mgl^{-1}).The PLBs for encapsulation were derived from shoot-tips cultured *in vitro*. Physical characteristics of the seeds 'beads' varied with the concentration of gelling agent and calcium chloride. Germination and regeneration potential of synthetic seeds under *in vivo* and *in vitro* conditions were assessed on different substrates. Among the different substrates tested for *in vitro* germination, best results were recorded in M alone and with AC supplemented with IAA, (1 mgl^{-1}); the seeds germinated with 93.50 ± 0.66% and 94.00 ± 0.61% frequency respectively, whereas under *in vivo* conditions in epiphytic compost 40.50 ± 0.43% seeds showed bead-to-plant conversion under *in vivo* conditions in epiphytic compost. Spherical, non-leaky, firm and self-breaking beads germinated efficiently and these could be stored for up to 90 days at 4°C temperature. The beads stored at ambient room temperature germinated with 49.00 ± 0.61% frequency after 15 days which was gradually reduced to 26.50 ± 0.82% and 0% after 30 and, 45 days respectively. The viability was completely lost after 45 days. The plantlets regenerated from encapsulated PLBs were subsequently hardened and established in green house conditions.

Introduction

IN CONTRAST to the rapid progress in micropropagation techniques, production of a large number of genetically identical plants is within the realm of reality. However, in the absence of an appropriate delivery and/or storage system for the regenerants, the practical application of such an advanced technology has remained limited. The possibility of encapsulating somatic embryos/ propagules in a nutritive gel, and using them for propagation purposes, is being increasingly realised (Murashige, 1978). Encapsulation is an important application of micropropagation technique, for the successful transfer of germplasm to distant laboratories and also establishing *in vitro* raised plantlets/seedlings to the field or greenhouse conditions. Synthetic seeds represent a novel system for exploiting the inherent polyembryonate potential of orchids (Vij *et al.* 1993, 2000). Encapsulation of somatic embryos or shoot buds and their subsequent development into complete plantlets has been reported in a number of species other than the orchids (Dutta *et al.*, 1999; Nayak *et al.*, 1998; Piccioni and Standardii 1995; Redenbaugh *et al.*, 1986). There are a few reports on the propagation of orchids using synthetic seeds (Dutta *et al.*, 1999, Martin, 2003; Mohanraj *et al.*, 2009; Nagnanda *et al.*, 2011; Pathak and Vij, 2005; Pehwal *et al.*, 2012; Sembi *et al.*, 2006; Vij *et al.*, 1993; Zhang *et al.*, 2009).

The technique improves the success rate of *in vitro* derived plantlets during laboratory to land transfer. Such capsules are useful in the exchange of germplasm between the laboratories due to their small size and relative ease in handling. Moreover, they ensure economy of space and aid in the germplasm conservation with proper preservation techniques (Piccioni and Standardii,1995). Presently, the efficacy of synthetic seeds was successfully tested in *Cymbidium aloifolium*. *Cymbidium aloifolium* is an Indo-Malayan, aloe-leaved elegant epiphytic taxon; which has long merited the attention of herbalist for its therapeutic properties. Besides being known for its floricultural significance, the species is very well known for its therapeutic uses. It is used to cure chronic illness, eye ailments, vertigo and paralysis. It is incorporated as one of the ingredients of an oil formulation to treat tumours which are both benign and malignant (Lawler, 1984). Due to habitat destruction and persistent collections for therapeutic and horticultural prominence, its natural populations are unremittingly getting scarce. As a consequence, the genus figures among the endangered orchids, enlisted in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2014). There is urgency to save its populations from getting extinct in the wild. Present effort is a step put forward in this direction to save the germplasm of this species using biotechnological means. This communication reports

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successful synthetic seed production and utilizing them in germplasm conservation and as an efficient propagation and delivery system. Successful attempts were made to directly transfer these synthetic seeds to the field following a sequence of treatments with bactericide, fungicide and growth regulator (IAA).

Materials and Methods

The shoot-tip derived PLBs (measuring 0.1-0.2 cm in length) were used to prepare synthetic seeds. In order to obtain desired number of propagules, the PLBs were multiplied in IAA (1mgl^{-1}) supplemented medium. They were subsequently cultured in hormone free Mitra *et al.*, (1976, M) medium for 3 wks to suppress subsequent multiplication cycles. The propagules were subjected to mild dehydration using filter papers (in Petri plates) at 25°C, by keeping them in a laminar air-flow cabinet.

Encapsulation

For encapsulation, 2-4% solutions of sodium alginate (CDH, Mumbai) was prepared in liquid M medium containing IAA (1mgl^{-1}; Hi-media, Mumbai). To accomplish complexation reaction, 25-100 mM $CaCl_2$ solutions were prepared in M medium containing IAA. For germination of the synthetic seeds under *in vivo* conditions, streptomycin (0.01% w/v) and bavistin (0.1% w/v) were added to the gel matrix. Both the gel matrix and complexation medium were autoclaved at 1.1kpa and 121°C for 20 minutes after adjusting the pH 5.7 with 1N-HCl and NaOH. The encapsulation was accomplished by mixing the PLBs in sodium alginate gel and pipetting them drop-wise, using a wide mouth pipette (10m), to a magnetically stirred (at 80 rpm) calcium chloride solution. The mixture was allowed to stand for 40 minutes. The beads, thus, prepared were thoroughly washed 2-3 times with sterilized double-distilled water prior to their storage.

Storage

The synthetic seeds were stored in the sterilized vessels (250 ml borosil flasks) at 4°C and 25°C. Their convertibility was tested at a fortnightly interval for 105 days on a variety of substratum (in the agarized medium, nutrient irrigated cotton).

Inoculation

Freshly prepared seeds were inoculated into 20 × 150 mm culture tubes which were maintained at 25 ± 2°C under 35 μ E m^{-2} s^{-1} light intensity and 50-60% relative humidity. One set of encapsulated PLBs were kept in the refrigerator at 4°C. Each treatment consisted of eight replicates. The experiment was repeated twice. Freshly prepared seeds were sown directly in the non-sterilized epiphytic compost to check their convertibility.

The plantlets raised from *in vitro* sown synthetic seeds were acclimatized stepwise; they were subjected to *in vitro* hardening by gradually starving them gradually of growth substances, carbon source, vitamins, micro-elements and macro-elements from nutrient mix at 15 days interval. The plantlets were deflasked and kept in loosely capped jars until the emergence of new growth and subsequently transferred to community pots in the greenhouse conditions under high relative humidity and low light in poly-tunnels. These were fertilized and watered regularly.

Results and Discussion

In the present experiment, synthetic seeds were successfully prepared in *Cymbidium aloifolium*. Their physical characteristics such as size, shape, firmness varied with the concentrations of the gelling agent and quantity of calcium chloride used. An encapsulation matrix of 3.5% sodium alginate and 75 mM $CaCl_2$ proved to be the best for the formation of firm, spherical-isodiametric; 0.8 cm, non-leaky beads (Table1; Fig.1). The lower concentrations of the (sodium-alginate; 2.0%. 2.5%, 3.0% and $CaCl_2$ 50 mM) were not suitable for encapsulation as the beads formed in these concentrations were irregularly outlined, very soft and leaky (Fig. 2). This could be due to the reduction in the gelling ability of the alginate after exposure to high temperature during autoclaving (Larkin *et al.*,1988). At high concentrations (sodium alginate; 4.0%, 4.5%, 5.0% and $CaCl_2$ 100 mM), the resultant beads were hard enough to cause delay in germination (Fig.3). Literature study reveals variable requirement of sodium alginate in earlier experiments *i.e.*, 1.5% - 2.0% (Sakamoto *et al.*, 1995); 2.0 - 3.0% (Redenbaugh *et al.*,1987); 5.0%-6.0% (Ahuja *et al.*,1989); 7.5% (Nagraj and Parkash, 1997) seems to be related to its batchwise efficacy and/or species specificity as has been hinted (Ahuja *et al.*,1989). It appears that the optimum concentration of sodium alginate varies with the quality of the chemicals, culture conditions and possibly the genotype as well. Presently, sodium alginate has been used as a gelling substance due to its property of solubility at room temperature, non-toxicity, low cost and easy availability and its ability to form permeable gel with $CaCl_2.2H_2O$, quick gellation, moderate viscosity (Onishi *et al.*,1992; Redenbaugh *et al.*, 1986). Interestingly, in the present experiment, the encapsulated propagules invariably proliferated under both, *in vitro* and *in vivo* conditions thus simulating the polyembryonate

Figs. 1-9. Conversion of synthetic seeds of Cymbidium aloifolium on different substratum: 1, Firm, spherical-isodiametric, non-leaky synthetic seeds; 2, Irregularly outlined, very soft and leaky beads; 3, Irregularly outlined, hard beads; 4-5, Profuse multiplication of PLBs inside the gel coat; 6-7, Differentiation of PLBs into hairy shoots and emergence of shoots out of the gel coat; 8, Complete plantlets; 9, Germination of synthetic seeds in the epiphytic compost.

Table 1. Effect of different concentrations of sodium alginate on organogenetic potential and texture of synthetic seeds of *Cymbidium aloifolium*.

Concentration of Sodium alginate (%)	Concentration of Calcium Chloride (mM)	Texture	Organogenetic potential
2.0	50	Irregularly outlined, leaky beads	-
2.5	50	Irregularly outlined, leaky beads	-
3.0	50	Soft beads, oblong shaped	-
3.5	75	Spherical, firm, non-leaky beads	+ + + +
4.0	100	Hard beads, oblong shaped	+
4.5	100	Very hard beads	-
5.0	100	Very hard beads	-

+, Fair response, + + + +, Satisfactory response; -, No response

potential of orchid seeds.

Earlier, self-breaking beads could be developed by immersing freshly formed seeds in magnesium and potassium nitrate solution with a view to make the seed resistant to suffocation inside the encapsulated propagule (Redenbaugh, 1986). Presently, the seeds were self-breaking without any pre-treatment and they converted with 93.50% and 94.0% frequency in M and M + AC medium respectively (Table 2). The PLBs in the encapsulated gel multiplied profusely (Figs. 4,5) and differentiated into hairy shoots (Figs. 6,7). The germination frequency in cotton irrigated by liquid medium epiphytic compost) was considerably impaired. The seeds were stored at two different temperature regimes, 4°C and 25°C (Table 3). At 25°C, the seeds germinated with only 49% frequency after 15 days of storage, which gradually reduced to 26.50% after 30 days. The viability was completely lost after 45 days of storage. At low temperature (4°C), the seeds could be stored for a period of 30 days without markedly affecting the frequency of bead-plant conversion. The low temperature was advantageous as nearly 90.0% seeds retained viability up to 30 days. This was possibly due to low metabolic rates at low temperatures in accord with earlier suggestion (Vij *et al.*, 2000). In general, the germination frequencies decreased with every passage of time. Such a decline has been reported due to the inhibited respiration of plant tissue by alginate coat (Redenbaugh *et al.*, 1987). High viability percentage and viability retention of stored artificial seeds at 4°C in comparison to room temperature indicated the efficiency of low temperature for storage and due to presence of nutrients inside the gel matrix.

Table 2. Conversion frequency of freshly formed synthetic seeds in *Cymbidium aloifolium* on different sowing substrata.

Sowing substratum	Conversion frequency (%)
M	93.50 ± 0.66
M + AC	94.00 ± 0.61
Cotton	10.75 ± 0.41
Epiphytic compost	8.75 ± 0.78
Epiphytic compost + B + S	33.25 ± 0.89
Epiphytic compost + B + S + 1AA	40.50 ± 0.43

M, Mitra *et al.*, (1976, M) medium; AC, Activated charcoal; IAA, Indole-3 acetic acid (1 mgl^{-1}); B, Bavistin; S, Streptomycin.

The direct transfer of seeds under *in vivo* conditions in epiphytic compost showed severe microbial contamination; the frequency of germination was very poor; only 8.75%. Different concentrations (0.01-0.05% w/v) of bactericide and fungicide (0.05-0.2% w/v) were tested and the optimum concentration was found to be 0.01% w/v of streptomycin and 0.1% w/v of bavistin. The regenerated plantlets (Fig.8) developed from synthetic seeds under *in vitro* conditions were successfully hardened. Incorporation of a bactericide (streptomycin; 0.01% w/v) and fungicide (bavistin; 0.1% w/v) in the gelling matrix proved useful to prevent the microbial contamination. Use of antimicrobial agents helped in checking the contamination but they reduced the poly-embryonate potential of the encapsulated PLBs which could be restored (up to 40.50%) by supplementing the gelling matrix with IAA at 1 mgl^{-1} (Fig.9). The use of antimicrobial agents was earlier proved to be beneficial for *in vivo* germination of the encapsulated shoot buds of *Valeriana wallichii* (Mathur *et al.*, 1989), somatic embryos of carrot (Sakamoto *et al.*, 1992)

Table 3. Conversion frequency of synthetic seeds at 4°C and 25°C.

Storage Period (days)	Conversion frequency (%) 4°C	25°C
0	93.00 ± 0.50	93.50 ± 0.66
15	91.00 ± 0.61	49.00 ± 0.61
30	90.00 ± 0.89	26.50 ± 0.82
45	86.25 ± 0.59	0.00
60	73.00 ± 0.75	0.00
75	48.75 ± 0.64	0.00
90	32.00 ± 0.93	0.00
105	0.00	0.00

sandalwood (Fernandes *et al.*, 1992) and encapsulated PLBs in *Spathoglottis plicata* (Nayak *et al.*, 1998).

The prime objective of devising an efficient protocol for the formation of synthetic seeds was to store species germplasm and to recover plantlets from them under *in vitro* and *in vivo* conditions. A sequential administration of bactericide and fungicide together with growth regulators assisted in improving the conversion rate of bead-to-plant under both conditions. In conclusion, the developed protocol provides direct transfer of synthetic seeds to external environment thus avoiding the time consuming process of hardening. Synthetic seeds technology provides a significant alternative to the perpetual maintenance of live materials for preservation of germplasm.

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References

Ahuja, P.S., J. Mathur, N. Lal, A.K. Mathur, and A. K. Kukreja. 1989. Towards developing artificial seeds by shoot bud encapsulation. *In: Tissue culture and Biotechnology of Aromatic Plants* (eds. A. K. Kukreja, A. K. Mathur, P. S. Ahuja, and A. K.Thakur) pp.20-78. CIMAP, Lucknow, India.

Dutta, K.B., B.Kanjilal, and D.D. Sarkar. 1999. Artificial seed technology: Development of a protocol in *Geodorum densiflorum* (Lam.) Schltr: An endangered orchid, *Curr. Sci.*, **76**:1142-45.

Fernandes, P.C., V.A. Bapat, and P.S. Rao. 1992. *In vivo* germination of encapsulated somatic embryos of *Santalum album* L. (Sandalwood), *Indian J. Exp. Biol.*, **30**:831-41.

Larkin, P. J., P. A. Davies, and G. J. Tanner. 1988. Nurse culture of low number of *Medicago* and *Nicotiana* protoplasts using calcium alginate beads. *Plant Sci.*, **58**:203-10.

Lawler, L. J. 1984. Ethnobotany of the orchidaceae- A manual. *In: Orchid Biology- Reviews and Perspectives.* Vol. III. (ed. J. Arditti) pp.27-149. Cornell University Press, Ithaca, New York, USA.

Mathur, J., P.S. Ahuja, N.Lal, and A.K. Mathur. 1989. Propagation of *Valeriana wallichii* using encapsulated apical and axial shoot buds. *Plant Sci.*, **60**:111-16.

Martin, K.P. 2003. Clonal propagation, encapsulation, and reintroduction of *Ipsea malabarica* (Reichb.f) Hook J.D.- An endangered orchid. *In Vitro Cell. Dev. Biol. Plant*, **39**:322.

Mitra, G.C., R.N. Prasad, and A.R.Chowdhury.1976. Inorganic salts and differentiation of protocorm in seed callus of an orchid and correlated changes in its free amino acid content. *Indian J. Exp. Biol.*, **14**:350-51.

Mohanraj, R., R. Ananthan, and V.N. Bai. 2009. Production and storage of synthetic seeds in *Coelogyne breviscapa* Lindl. *Asian J. Biotechnol.*, 1:124-28.

Murashige, T. 1978. The impact of plant tissue culture on agriculture. *In*: *Frontiers of Plant Tissue Culture* Intl. Association for Plant Tissue Cultures (ed. T. Thorpe). pp. 15-16. University of Calgary, Canada.

Nagraj, S. and D. Prakash. 1997. Encapsulation and regeneration of protocorms of *Vanda teres*. *In*: *Proc. Natl. Seminar on Developmental Biology and Commercialization of Orchids*. (ed. S.P. Vij) pp. 101. The Orchid Society of India, Panjab University, Chandigarh, India.

Nagananda, G. S., N. Satishchandra, and S. Ragath. 2011. Regeneration of encapsulated protocorm like bodies of medicinally important vulnerable orchid *Flickingeria nodosa* (Dalz.) Seidenf. *Int. J. Bot.*, 7:310.

Nayak, N. R., S.P. Rath, and S.N. Patnaik. 1998. High frequency plant regeneration from alginate encapsulated protocorm-like bodies of *Spathoglottis plicata* Bl., A terrestrial orchid, *Phytomorphology*, **48**:179-86.

Onishi, N., T. Mashika, and A. Okamoto. 1992. Cultural system producing encapsulable units of synthetic seeds in celery. *Acta Hortic.*, **319**:113-18.

Pathak Promila and S.P. Vij. 2005 Regeneration of *Aerides multiflora* Roxb. using synthetic seeds: A study *in vitro*. *In: Proc. 17th World Orchid Conference. Shah Alam.* 2002 (eds. J. Arditti and Halen Nair) pp. 267-71. Borneo Natural History Publication, Kota Kinabalu, Sabah, Borneo, Malaysia.

Pehwal, A., S.P. Vij, Promila Pathak, and L.K. Attri. 2012. Augmented shelf-life and regeneration competence of AC supplemented synthetic seeds in *Cymbidium pendulum* (Roxb.) Sw. *Curr. Bot.*, 3:30-34.

Piccioni, E. and A. Standardii. 1995. Encapsulation of micropropagated buds of six woody species. *Plant Cell Tissue Organ Cult.*, **42**:221-26.

Redenbaugh, K. 1986. Analogs of botanic seeds, *U.S Patent*.

4:562-663.

Redenbaugh, K., B.D. Pasch, J. W. Nichol, M. E. Kossler, P. R. Viss, and K.A. Walker. 1986. Somatic seed: Encapsulation of asexual plant embryos. *Biotechnology*, **4**:797-801.

Redenbaugh, K., D. Slade, P. Viss, and J. Fujii. 1987. Encapsulation of somatic embryos in synthetic seed coats. *Hort. Sci.*, **22**:803-09.

Sakamoto, Y., N. Orishi, and T. Hirosawa. 1995. Delivery systems for tissue culture of encapsulation, in automation and environmental control in plant tissue culture (ed. J. Aitken-Christie, T. Kozai. and M. Lila Smith) pp. 215-43. Kluwer Academic Press, Netherlands.

Sakamoto, Y., T. Mashiko, A. Suzuki, H. Kowata, and A. Jwasaki. 1992. Development of encapsulation technology for synthetic seeds. *Acta Hortic.*, **319**:71-76.

Sembi, J.K., J. Verma, S. Aggarwal, Promila Pathak, and S.P. Vij. 2006. *In vitro* propagation of *Aerides multiflora* Roxb, (Orchidaceae) through encapsulated PLBs. *J. Orchid Soc. India*, 20:21-23.

Vij, S.P., A. Kher, and Ashish Gupta. 2000. Orchid micropropagation. *In*: *Biotechnology in Horticultural and Plantation Crops*. (eds. K.L. Chadha, P.N. Ravindran, and L. Sahijram) pp. 598-641. Malhotra Publishing House, New Delhi, India.

Vij, S.P., Promila Pathak, P. Kaur, and V. Sharma. 1993. Somatic/artificial seeds in Orchids. *Orchid News*, **8-9**:12-13.

Zhang, M., S. Peng, X. Yang, and L. Xu. 2009. Preparation technique of *Cremastra appendiculata* synthetic seed. (in Chinese with English abstract), China. *J. Chinese Materia. Med.*, **34**:1894-97.