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In vitro Conservation and Exploiting Polyembryonate Potential of Synthetic Seeds of *Malaxis acuminata* D. Don

Saranjeet Kaur*

Department of Botany, Panjab University, Chandigarh, India

Key words: Conservation, Endangered, *in vitro*, Protocorms, Synthetic seeds

Abstract
Synthetic seeds were prepared in *Malaxis acuminata* by encapsulating shoot tip derived protocorm-like bodies (PLBs; 0.2-0.5 cm) in a hydrated gel capsule composed of calcium alginate matrix (sodium alginate; 3.5% and calcium chloride; 75 mM) in liquid MS medium. Synthetic seeds varied in physical features with the concentration of gelling agent and calcium chloride. In *in vitro* germination, response was achieved in MS medium fortified with BAP (1 mg/l); where synthetic seeds germinated with 100% frequency. Physically firm, globular, non-leaky, and self-breaking beads germinated efficiently which could be stored for up to 90 days at 4°C. At room temperature, synthetic seeds germinated with 50% frequency after 15 days and the germination frequency steadily reduced to 0% after 45 days of storage. Under *in vivo* conditions, synthetic seeds showed 80% bead-to-plant conversion in MS medium supplemented with antibacterial, antifungal agents and BAP (1 mg/l).

Introduction
Malaxis acuminata is a hemicolos orchid species. Endemic to the tropical Himalayas, this pseudobulbous species is scattered in pine forests, at an altitude of 1800-2300 meter. This herbaceous plant also called 'Rishbhak', is recognized for its therapeutic worth as it is highly enriched with β-sitosterol compounds (Pushpa et al. 2011). Its dry pseudobulbs are used as a component of 'Ashtavarga' in ayurvedic medicine 'Chyavanprash' (cf. Chauhan 1990). The species also possess anti-tuberculosis, aphrodisiac, anti-inflammatory and anti-aging properties (Bose et al. 2017). Due to decline in the wild populations of *M. acuminata*, it has been identified as a rare species and included in the Appendix II of CITES (2022) among other plant species. Scientific strategy must be adopted to save this species from getting extinct in its wild ever-decreasing reserves. Synthetic seed technology is an innovative technique that exploits the intrinsic poly-embryonate potential of *in vitro* raised propagules further establishing *in vitro* produced

*Author for correspondence: <sarana_123@rediffmail.com>. Present address: Department of Biosciences, University Institute of Biotechnology, Chandigarh University, Gharuan, District- Mohali, Punjab, India.

plantlets, in their habitats. The technique also promises for easy transference of germplasm to far-off laboratories as synthetic seeds are small in size and occupy less space. In orchids, synthetic seeds are quite beneficial as the encapsulation of non-endospermic seeds and other propagules into alginate capsule allows them to germinate like endospermic seeds (Kumar and Loh 2012). Synthetic seed technology helps in enhancing the survival of *in vitro* formed plantlets during laboratory to land transfer besides ensuring space economy (Piccioni and Standardii 1995); these are also used in cryopreserving orchid species for instance *Orchis morio*, *Dactylorhiza fuchsia* (Wood et al. 2000); *Diuris arenaria* and *Pterostylis saxicola* (Sommerville et al. 2008). Synthetic seeds are also advantageous for the propagation of such plants that do not produce seeds, polyploids having selective traits and transgenic plants. Synthetic seeds remain viable upon storage for considerable time period besides multiplying the gemplasm (Capuano et al. 1998). In literature, there are few reports available on propagation of orchid species using synthetic seeds (Dutta et al. 1999, Martin 2003, Mohan et al. 2009, Nagananda et al. 2011, Pathak and Vij 2005, Pehwal et al. 2012, Vij et al. 1993, Zhang et al. 2009, Kaur and Pathak 2014, Bektas and Sokmen 2016). So, attempts were made to prepare synthetic seeds by encapsulating PLBs in calcium alginate gel and the effect of bactericide, fungicide and growth regulator BAP (1 mg/l) was also assessed.

Materials and Methods

The PLBs were obtained from *in vitro* shoot-tip culture. PLBs were multiplied in cytokinin (BAP at 0.5 mg/l) supplemented MS medium. These were cultured in hormone free MS medium for nearly 3 weeks initially. Inside a laminar air-flow, the propagules were mildly dehydrated using filter paper folds (in petri plates) at 25°C. For encapsulation of PLBs, sodium alginate (2-4%; CDH, Mumbai, India) solutions of varying consistencies were prepared in liquid MS medium fortified with BAP (1.0 mg/l; Hi-media, Mumbai, India). Calcium chloride ($CaCl_2$) solution of varying concentrations (i.e. 25-100 mM) was prepared in MS medium for complexation process. In separate set of experiment, streptomycin (0.01% wv^{-1}) and bavistin (0.1% wv^{-1}) were also incorporated into the gel matrix to check bacterial and fungal infection during germination of synthetic seeds in *in vivo* conditions. Both nutritive gel and complexation medium were autoclaved at 121°C and 1.1 kpa for 20 minutes after adjusting their pH at 5.7 with 1N-HCl and NaOH. The PLBs were mixed in sodium alginate gel matrix pipetted drop-wise with the help of wide-mouth pipette (10 mm), in a magnetically stirred (at 80 rpm) solution containing calcium chloride. Further, mixture was allowed to stand for 40 minutes. Finally, synthetic seeds were thoroughly washed 2-3 times with sterilized double-distilled water prior to their storage. The freshly prepared synthetic seeds were stored in the sterilized vessels (250 ml flasks and test tubes) (Borosil, India, Limited) at 4°C and 25°C. Their convertibility was tested at fortnightly interval for up to 105 days. Freshly formed synthetic seeds were inoculated into culture tubes (20 × 150 mm size) containing agar solidified MS medium. The culture vessels were incubated at 25 ± 2°C under 40

$\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and 50-60% relative humidity. One set of encapsulated PLBs was stored in a refrigerator at 4°C. Each treatment consisted of eight replicates. Reproducibility of the experiment was checked by repeating the experiment twice. The experiment was set up using complete randomized design (CRD) with eight replicates per treatment. The effect of sowing substratum on conversion frequency and time taken to regenerate in weeks was tested using Tukey's multiple comparison test ($P \leq 0.05$) in one way ANOVA. The statistical analyses were performed using SPSS (version 17) software package (SPSS Inc., Chicago, USA). The results are expressed as mean ± SD of eight replicates.

Results and Discussion

The alginate gel acts as an endosperm by augmenting nutrition to the encapsulated propagule. Presently, synthetic seeds were prepared in *M. acuminata*. Their physical features such as shape, size, and firmness differed with varying concentrations of the gelling agent and amount of calcium chloride used. Sodium alginate (at 3.5% conc.) and $CaCl_2$ (at 75 mM conc.) proved to be optimum for the formation of spherical-isodiametric (0.8 cm), non-leaky and firm beads (Fig. 1a). Sodium-alginate at other concentrations i.e. 2.0, 2.5 and 3.0% and $CaCl_2$ at 50 mM was not found suitable for encapsulating the propagules. The beads formed in above mentioned concentrations were leaky, very soft and irregularly outlined (Fig. 1b). On the other hand, the high concentrations of sodium alginate (4.0, 4.5 and 5.0%) and $CaCl_2$ (100 mM) resulted into hard coat formation of synthetic seeds (Fig. 1c). Lower concentrations of sodium alginate showed decline in the gelling ability of the alginate matrix that possibly could have occurred due to exposure to extremely high temperature of gel matrix while autoclaving (Larkin et al. 1988). A survey of literature indicated the variable requirements of sodium alginate matrix i.e., 1.5 - 2.0% (Redenbaugh et al. 1987), 2.0 - 3.0% (Ahuja et al. 1989), 5.0 - 6.0% (Nagraj and Prakash 1997), 7.5% (Onishi et al. 1992) which seems to be linked to the batchwise efficacy and / or species specificity as already been indicated (Ahuja et al. 1989). Now a days, sodium alginate has been used as a gelling medium because of its quick gelling, less cost, easy availability, non-toxicity, and ability to form permeable gel with $CaCl_2.2H_2O$, and its solubility at room temperature (Redenbaugh et al. 1986, Saiprasad 2001). Self-breaking beads were formed that converted with cent percent frequency in MS medium. The propagules proliferated inside gel capsule (Fig. 1d). The proliferative potential of propagules was increased in BAP augmented MS medium (Table 1; Fig. 1e and 1f). In earlier experiments, self-breaking beads were formed by immersing freshly formed synthetic seeds in magnesium and potassium nitrate solution with a view to make them suffocation resistant from inside the encapsulated coating of the propagule (Redenbaugh, 1986). Synthetic seeds were stored at two different temperatures i.e. 4°C and 25°C (Fig. 2). At 25°C, synthetic seeds germinated with a lower frequency i.e. 50.00 % after 15 days of storage which further declined by another 25% after 30 days of storage. Viability was completely lost upon storage for 45 days. At lower temperature of 4°C, the seeds could be

stored for longer periods (90 days) without rapid loss of viability. The low temperature was observed to be more advantageous as synthetic seeds retained their viability even up to 90 days. Low temperature was advantageous as all seeds retained viability up to 90 days probably due to their reduced metabolic rates at low temperatures. The results are in accord with earlier findings (Kaur and Pathak 2014, Vij et al. 2000, Mathur et al. 1989). Further, a decline in germination frequency was also observed with every passage of time. Such a decline in the conversion frequency has been attributed to the altered

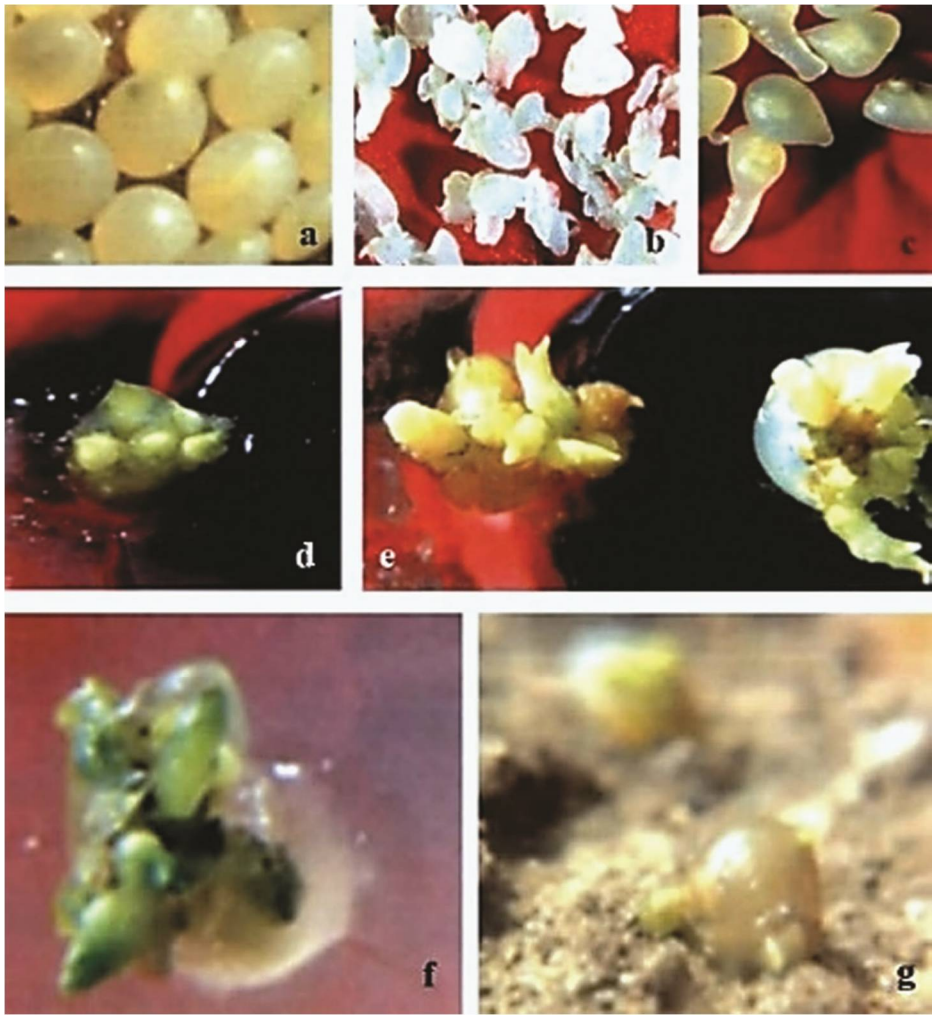

Fig. 1. Synthetic seeds of *Malaxis acuminata*. (a) Spherical-isodiametric (0.8 cm), non-leaky and firm beads. (b) Leaky, very soft and irregularly outlined. (c) Hard coat formation of synthetic seeds. (d) Proliferation of the propagules in MS medium inside gel capsule. (e), (f) Proliferation of the propagules in MS + BAP medium. (g) Antimicrobial agents restored the poly-embryonate potential of encased PLBs.

physiological process i.e. inhibition of respiration of the plant tissue by alginate coating (Ahuja et al. 1989). Retention of high percentage of viability in stored synthetic seeds at 4°C in contrast to 25°C (room temperature) indicated the efficacy of low temperature for storage purpose and presence of nutrients inside the gel matrix. Under *in vivo* conditions the synthetic seeds when sown in sand irrigated with water, not only showed reduced germination frequency of 25 per cent but the propagules got contamination as well (Table 1).

Table 1. *In vitro* and *in vivo* conversion and poly-embryonate potential of encapsulated PLBs of *Malaxis acuminata*.

Conversion medium	% Conversion frequency		Embryogenic Potential
	Under *in vitro*	Under *in vivo*	
MS	65.00 ± 0.00^{c}	-	2 PLBs
MS + BAP$_{(1)}$	100.00 ± 0.00^{b}	-	6-7 PLBs
MS$^{\#}$	70.00 ± 0.05^{a}	-	1 PLBs
Sand + distilled water	-	25.15 ± 0.08^{a}	No multiplication
MS$^{\#}$ + BAP $_{(1)}$ + Sand + B+S	-	50.00 ± 0.25^{b}	3-4 PLBs

MS$^{\#}$, Murashige and Skoog medium devoid of sucrose; B, Bavistin; S, Streptomycin at 1mg l^{-1}.Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test.

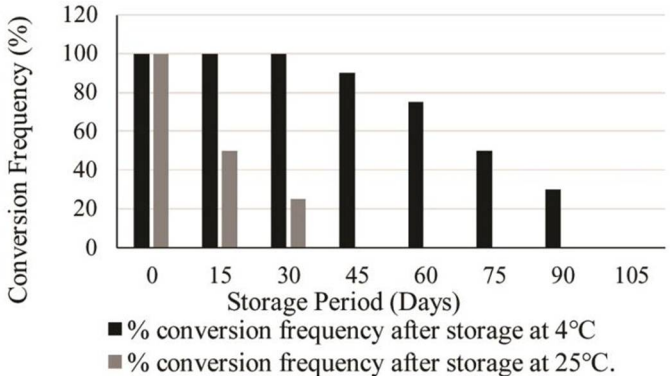

Fig. 2. Conversion frequency of synthetic seeds of *Malaxis acuminata* stored at 4 and 25°C.

In order to reduce contamination, synthetic seeds were irrigated with MS medium bereft of sucrose along with bactericide (0.01-0.05% wv^{-1}) and fungicide (0.05-0.2% w/v^{-1}) of the optimum concentration of 0.01% wv^{-1} of streptomycin and 0.1% wv^{-1} of Bavistin; the regeneration response was elevated up to 50%. Incorporation of antimicrobial agents successfully checked microbial infection and restored the poly-embryonate potential of encased PLBs (Fig. 1g). A perusal of literature reveals that the results are in accord with similar earlier findings where inclusion of antimicrobial agents, in the nutrient pool, proved beneficial for *in vivo* germination of encapsulated shoot buds of *Valeriana wallichii*

(Sakamoto et al. 1992, Kaur and Pathak 2014), somatic embryos of carrot (Sakamoto et al. 1992, Fernandes et al. 1992) and encapsulated PLBs in *Spathoglottis plicata* (Nayak et al. 1998). The incorporation of growth regulators in the gelling matrix increases the germination capacity, viability and enhance the storage capacity of synthetic seeds (Redenbaugh 1986). However, the addition of bactericide and fungicide with growth regulator improved bead-to-plant conversion rate under *in vivo* conditions.

Synthetic seeds are used as a tool to conserve rare, endangered medicinal plants of commercial value. Synthetic seeds technology is a significant alternative for the conservation of germplasm. The globular, self-breaking, physically firm, and non-leaky synthetic seeds germinated readily as compared to those synthetic seeds which were made at varying concentrations of gelling agent and calcium chloride. The incorporation of antibacterial and antifungal agents and growth regulator multiplied the propagules inside gel.

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