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Ex situ conservation of *Dendrobiumnobile* through encapsulatedprotocorm-like bodies

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Abstract

Synthetic seeds 'beads'were prepared in Dendrobiumnobileby encapsulating protocorm like bodies (PLBs ; 0.1-0.2 cm) in a hydratedgel case made up of calcium alginate (Sodium alginate at 3.5% and Calcium chloride at 75 mM) matrix inliquid Mitraet al., 1976 (M) medium. For encapsulation, the protocorm like bodies, were obtained from in vitro cultured shoot-tips. Physical features of synthetic seeds differed with the concentration of gelling agent and calcium chloride taken.Germination potential of synthetic seeds under in vivo and in vitro conditions was assessed on different substrates. Among the variety of substrates tested for in vitro germination, optimum results were recorded in M medium alone andfortified with BAP.The seeds germinated with 100% frequency. Firm,globular, non-leaky, and self-breaking beads germinated efficiently.These could be stored for up to 90 days at 4 ℃temperature. The beads stored at room temperature, germinated with 75% frequency after 15 days which steadily reduced to 0% after 75days of storage respectively.The plantlets regenerated from encapsulated PLBs were subsequently established in the green house conditions. Under in vivo conditions, epiphytic compost with antibacterial, antifungal agents and with BAP favoured 80% bead-to-plant conversion.

Keywords**:** *conservation, endangered, in vitro, protocorms, synthetic seeds*

1.Introduction

*Dendrobiumnobile*Lindl. is an epiphytic, sympodial orchid species. Biogeographically, it is distributed from south China, Laos, Thailand to the North-Eastern Indian Himalayas. The species is scattered in the tropical to temperate region, at an altitude of 200-2000 meters. *D. nobile* is a floriculturally significantspecies and a progenitor of numerouscommendable hybrids of international status. Besides possessing a great ornamental potential, the species is medicinally important as well.The herbal species is known to be one of the 50 fundamental herbs that are used in the traditional system of chinese medicine. *D. nobile* known as shihu or shihulan is of extreme worth [1]. The drug is used, as a strengthening tonic, aphrodisiac, known to nourishes the yin-system of body, enhanceslongevity; also its stems are useful in quenching thirst, calming restlessness, accelerating convalescence and also reducing the dryness of mouth [2].

Since the species is extremely versatile in possessing ornamental and medicinalvalues, its natural populations are continuously and speedily getting rare due to indiscriminate collections and habitat destruction due to deforestation, fragmentation of habitat especially in the tropical regions, which far exceed its natural regeneration, increased use of fertilizers, excessive exploitation of the soil adds up to its current rare, endangered and threatened (RET) status.Infact, the genus *Dendrodium*is included in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora [3].Scientific methodologies are required to save the species from getting extinct in nature. Consequently, through this communication,it is emphasized to propagate, multiply, and conserve the species using tissue culture techniques that assists with the conservation of *D nobile*.

In vitro culture techniques offer alternative pathways to generate the significant number of seedlings/plantlets within a short span of time. Synthetic seed technology is a novel technique for exploiting the inherent poly-embryonate potential of *in vitro* raised propagules besides establishing the *in vitro* raised plantlets in the natural environment.The technique isuseful in conservation of elite, endangered genotypes besides providing easy transportation to distant laboratories due to their small size and relative ease in handling.

The *in vitro* raised plantlets are extremely vulnerable to desiccation and attack of pathogens when exposedto natural conditions. Their susceptibility becomes a hindrance whilemechanically planting themin the field or greenhouse conditions.The technique increases the success rate of *in vitro* derived plantlets during laboratory to land transfer shift. Moreover, synthetic seeds ensure economy of space and help in conserving thegermplasm throughscientific techniques [4].

Synthetic seeds are also successfully utilized in cryopreservation of various orchid species such as *Dactylorhiza fuchsia*, *Orchismorio*[5]; *Pterostylissaxicola*, *Diurisarenaria*[6].

The synthetic seeds are also advantageous for the propagation of transgenic plants, polyploids with selected traits, and non-seeded plants that cannot be propagated through seeds. These seeds are also considered to be an alternative source for propagating several economically and commercially important genotypes. This efficient technique promises to multiply germplasm*in vitro* as synthetic seeds are able to retain their viability upon storage for certain time period [7] (Capuano et al.1998).There are a few reports on the propagation of orchids using synthetic seeds [8][9][10][11][12][13][14][15][16][17]

Present attempt is a step forward to utilize the synthetic seed technology to conserve*D. nobile*. Successful attempts were made to transfer these synthetic seeds straightaway to the soil following a sequence of treatments with bactericide, fungicide and growth regulator (BAP). Through this communication, it is stressed to conserve, propagate and multiply the species, thus, saving its wild populations from getting extinct.

2.Materials and Methods

The PLBs (measuring 2 mm in length), obtained from *in vitro*shoot-tip culture, were utilized to prepare synthetic seeds. To get more number of propagules, the protocorm-like bodies were multiplied in benzyl amino purine(BAP at 0.5 mgl^{-1}) supplemented M medium. They were further cultured in hormone bereft M medium [18] for nearly 4wks to suppress subsequent cycles of PLB multiplication. The propagules were mildly dehydrated by keeping them in filter paper folds (in petri plates) at 25℃ inside a laminar air-flow cabinet.

2.1 Encapsulation

To encapsulate the protocorm-like bodies (PLBs),sodium alginate (2 - 4%; CDH, Mumbai,India) solutionsof varying consistencies were prepared in liquid M medium fortified with BAP (0.5 mgl^{-1}; Hi-media, Mumbai, India). Calcium chloride($CaCl_2$) solution of varying concentrations (i.e. 25-100 mM) was prepared in M medium containing (BAP, 1.0 mgl^{-1}) for complexation process. In separate set of experiment, the antibacterial and antifungal streptomycin (0.01% wv^{-1}) and bavistin (0.1% wv^{-1}) were also incorporated into the gel matrix to check infection during germination of synthetic seeds in *in vivo* conditions. Bothnutritive gel and complexation medium were autoclaved at 121℃and1.1kpa for 20 minutes after adjusting their pH at 5.7 with 1N-HCl and NaOH. The encapsulation was achieved by mixing the PLBs in sodium alginate gel matrix. The gel with PLBs was pipette-out drop-wise using a wide mouth pipette (10mm), to a magnetically stirred (at 80 rpm) calcium chloride solution. Later, the mixture was allowed to stand for 40 minutes. Finally, the synthetic seeds were thoroughly washed 2-3 times with sterilized double-distilled water prior to their storage.

2.2 Storage

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

2.3Inoculation

The freshly formed seeds were inoculated into 20 × 150 mm culture tubes. The culture vessels were maintained at 25 ± 2℃ under 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and 50-60% relative humidity. One set of encapsulated PLBs was maintained at 4℃ in the refrigerator. Each treatment consisted of eight replicates. The experiment was repeated twice. Also, the freshly prepared seeds were sown straightaway in the non-sterilized epiphytic compost to check their convertibility. The plantlets formed from *in vitro* sown synthetic seeds were given stepwise acclimatization treatment. They were hardened *in vitro* by gradually starving them of growth regulators, carbon source, vitamins, micro-elements and macro-elements from nutrient pool at 15 days interval. Later, the plantlets were deflasked and kept in loosely capped jars until the emergence of new growth. Finally, they were transferred to community pots in the greenhouse under high humidity and low light conditions in poly-tunnels. These were fertilized and irrigated regularly.

2.4Observations and statistical analysis

The experiment was set up using complete randomized design (CRD) with eight replicatesper treatment. The effect of sowing substratum on conversion frequency and time taken to regenerate in weeks to form complete plantlets was tested usingTukey's multiple comparison test ($P \leq 0.05$) in one way ANOVA to separate of significantly different groups. The statistical analyses were performed using the SPSS (version 17) software package. (SPSS Inc., Chicago, USA).The results are expressed asmean ± SD of eight replicates.

3. Results and discussion

Presently, the synthetic seeds were successfully prepared in*Dendrobiumnobile*. Their physical features such as shape, size, and firmness varied with different concentrations of the gelling agent and amount of calcium chloride used. The concentration 3.5% sodium alginate and 75 mM $CaCl_2$ proved to be the optimum for the formation of spherical-isodiametric (0.8 cm), non-leaky and firm beads (Figure 1a). Sodium-alginate at other concentrations i.e. 2.0%, 2.5%, 3.0% and $CaCl_2$ at 50 mM was not found suitable for encapsulating the propagules The beads formed in abovementioned concentrations were leaky, very soft and irregularly outlined (Figure 1b). The decline in the gelling ability of the alginate matrix possibly could have occurred due to exposure to extremely high temperature while autoclaving [19]. On the other hand, the high concentrations of sodium alginate (4.0%, 4.5%, 5.0%) and $CaCl_2$ (100 mM) resulted into hard coat formation of synthetic seeds that might have caused delay in germination although the seeds appeared roundin shape (Figure 1c). A survey of literature indicates about the variable requirements of sodium alginate matrix *i.e.,* 1.5% - 2.0% [20]; 2.0 - 3.0% [21]; 5.0% - 6.0% [22]; 7.5% [23] which emerges to be related to the batchwise efficacy and/or species specificity as has been hinted [21].
In the present experiment, before encasing PLBs in the nutrient gel they were desiccated. Literature study indicates that desiccation maintains the propagule in a quiescent state for extended periods of time if the somatic embryo is dried to moisture content of approximately 10%, as in a number of true seeds, probably increasing the viability of the seeds upon storage.

Presently, sodium alginate has been used as a gelling medium due to its low cost, easy availability, quick gellation, non-toxicity, ability to form permeable gel with $CaCl_2.2H_2O$ moderate viscosity, and solubility at room temperature [24][25]. Under *in vitro* and *in vivo* conditions, the propagule inside the synthetic seeds proliferated, thus, simulating the polyembryonateability of orchid seeds. They germinated on agarized medium without ; (Figure1d) and with AC fortified medium and multiplied profusely inside the gel capsule with cent per cent frequency (Table 1). Literature survey indicates similar efficacy of additional activated charcoal in the gelling matrix enhancing multiplication of the propagules[26]. Presently, it was made possible to develop self-breaking beads without giving any pre-treatment. These self-breaking beads converted with cent percent frequency in M medium (Table 2). 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 insidethe encapsulated coating of the propagule[26]. The multiplication potential was accentuated in the presence of additional BAP; the protocorms multiplied profusely inside the alginate envelope (Figure 1e)and developed into healthy plantlets (Figure 1f).The conversion frequency was considerably impaired in cotton and epiphytic compost (Table1; Figure 1g) irrigated by liquid M medium similar to those reported earlier[27][16].Theseeds were stored at two different temperature regimes i.e. 4℃ and 25℃ (Figure 2).

The seeds stored at 25℃ germinated with a reduced frequency i.e. 50.00 % after 15 days of storage.The conversion frequency further declined to 25 % after 30 days of storage period. The seeds completely lost viability upon storage for 45 days. At4℃, the seeds could be stored for longer periods (30 days) without rapidly losing viability and did not show much decline in the bead-plant conversion frequency. The low temperature was advantageous as all of the beads retained viability up to 30 days. This could have been possible due to their reduced metabolic rates at low temperatures. The results are in accord with earlier findings [16] [27][28].In the present experiment, a decline in the germination frequency was observed with every passage of time. Such a decline in the conversion frequency has been attributed to the altered physiological process i.e. inhibition of respiration of the plant tissue by alginate coating [21]. Retention of high percentage of viability in stored synthetic seeds at 4℃ in contrast to 25℃ (room temperature) indicated the efficacy of low temperature for storage purpose and presence of nutrients inside the gel matrix (Figure 2).

Under *in vivo* conditions in the epiphytic compost, synthetic seeds showed high microbial contamination and declined germination percentage (i.e. 25 %).In order to reduce the contamination

Different concentrations of bactericide (0.01- 0.05% wv^{-1}) and fungicide (0.05-0.2%w/v^{-1}) were tested; their optimum concentration was found to be 0.01% wv^{-1} of streptomycin and 0.1% wv^{-1} of bavistin.During hardening the incorporation of antimicrobial agentssuccessfully checked any microbial infection and restored the poly-embryonate potential of the encased PLBs (up to 80%) by fortifying the gelling mix with BAP at $1mgl^{-1}$(Figure 1h, 1i)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 *Valerianawallichii*[29][16], somatic embryos of carrot [29] [30] and encapsulated PLBs in *Spathoglottisplicata*[31].The incorporation of growth regulators in the gelling matrix increases the germination capacity, viability and enhance the storage capacity of artificial seeds [26].The addition of bactericide and fungicide in conjunction with growth regulator improved bead-to-plant conversion rate under *in vivo* conditions.

4. Conclusion

The main objective aim of the experiment was to conserve the germplasm of *D nobile* under *in vitro* conditions besides establishing the plantlets by transferring the synthetic seeds to external environment.The incorporation of antibacterial and antifungal agents together with

growth regulator assisted with the conversion of beads into plantlets. Synthetic seeds technology makes available a significant alternative for preservation of the germplasm.

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***Table* 1 Conversion frequency of freshly formed synthetic seeds in *D. nobile* on different sowing substrata.**

Sowing substratum	Conversion frequency (%)		Complete plantlets (wks)
	Under *in vitro*	Under *in vivo*	
M	100.00±0.00[a]	-	16.10±0.01[a]
M + BAP	100.00±0.00[a]	-	16.05±0.05[a]
M + Cotton	80.00±0.01[b]	-	17.00±0.59[ab]
M* + Epiphytic compost	-	25.15±0.08[c]	20.06±0.00[c]
M*+ Epiphytic compost+B+S	-	67.00±0.08[ab]	15.12±0.13[a]
M*+ Epiphytic compost+B+S+BAP		80.00±0.00[a]	15.06±0.02[a]

M, Mitra*et al*., (1976, M) medium; M*, Mitra*et al*., (1976, M) medium devoid of sucrose; B,Bavistin; S, Streptomycin; BAP at1mg l^{-1}

Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test.

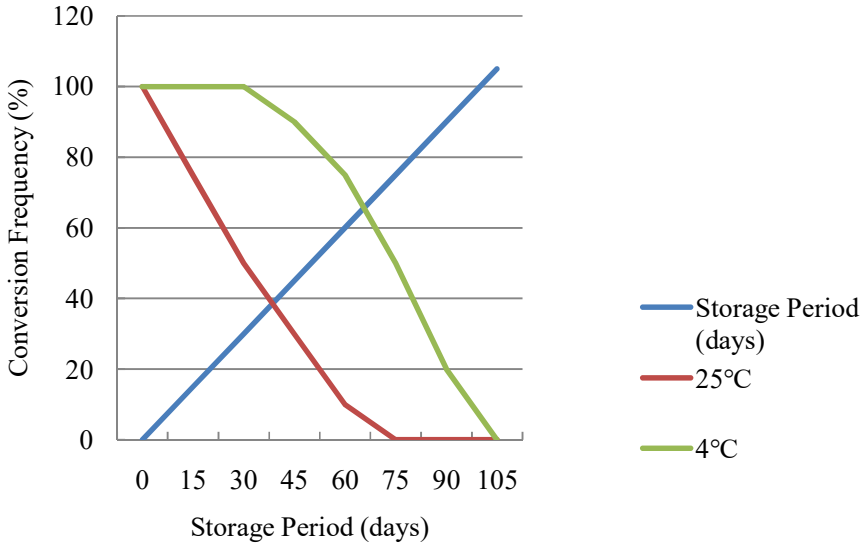
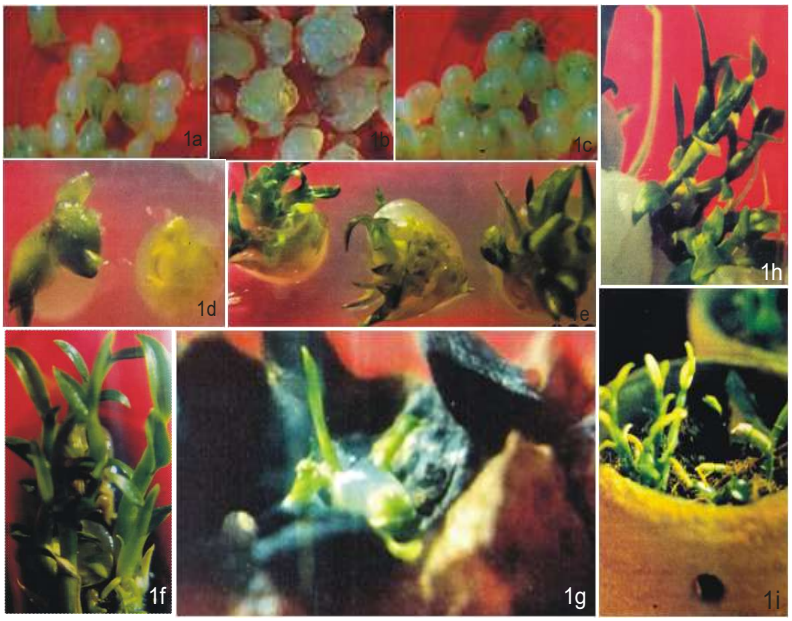


Figure 2 Conversion frequency of synthetic seeds of *D. nobile* at 25°C and 4°C



Explanation to the figures

Figure 1 a-i *Ex situ* conservation of *Dendrobiumnobile* through encapsulated protocorm-like bodies, a. spherical-isodiametric, non-leaky and firm beads, b. irregular shaped leaky beads, c. hard beads, d. sprouting of the synthetic seed, e. Multiplication of the propagule inside the gel coat, f. plantlet development from self breaking beads, g. synthetic seed in epiphytic compost, h-i hardening of the plantlets.