

**Studies on the production of synthetic seed of  
*Cymbidium elegans* x *C. aloifolium* hybrid**

A dissertation

Submitted in partial fulfillment of requirement

for the award of the degree of

**MASTER OF TECHNOLOGY**

**in**

**BIOTECHNOLOGY**

**2015**

**Under The Guidance of**

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July 2015

## DECLARATION

I, under designated, hereby declare that the research work presented in the M.Tech dissertation entitled "**Studies on the production of synthetic seed of *Cymbidium elegans* x *C. aloifolium* hybrid**" has been carried out by me under the supervision and guidance of **Dr. Anil Kumar**, Associate professor, Department of Biotechnology, Thapar University, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any university or examining body in India/ elsewhere.



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## CERTIFICATE

This is to certify that dissertation entitled, "**Studies on the production of synthetic seed of *Cymbidium elegans* x *C. aloifolium* hybrid**" submitted by Ms. Nitika Vats in partial fulfillment of the requirement for the award of M.Tech in Biotechnology at Thapar University, Patiala is an authentic work carried out by her under our supervision and guidance.

To the best of our knowledge, the matter embodied in this dissertation has not been submitted to any other university/ Institute for award of any Degree or Diploma.



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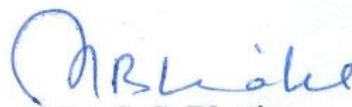
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## ACKNOWLEDGMENT

In the praise of almighty who bestowed me the divine guidance to embark upon this task of keeping in realms of facts and events. The period of my thesis work has proved to be the most exciting phase of my life and now I believe that you are never given a wish without also being given the power to make it true you have to work for it however what seemed to be daunting and uphill task is now nearing completion. This has largely been made possible due to my association with a number of people. My utmost gratitude goes to my esteemed advisor **Dr. Anil Kumar, Associate Professor, Department of Biotechnology, Thapar University, Patiala**, who greatly enriched my knowledge & constantly inspired me and who, in my pursuit of this thesis, had to sacrifice many of his precious hours. He has been able to skillfully manage this research without forgetting the ever essential human relationships. From deep inside thank you and your family for their opportune support and help. I wish to express my deep sense of humbleness to **Dr. Dinesh Goyal, Head of the Department of Biotechnology, Thapar University, Patiala, Punjab**. A special thanks to all faculty members for their constant encouragement and support throughout the project work. Indeed the words to my command are not adequate either in form or in spirit to convey my in depth feelings of gratitude to **Ms. Amanpreet Kaur** for her valuable guidance, incessant encouragement, constructive criticism, expedient advice and affection during the course of investigation and preparation of this manuscript. I feel lacunae of words to express my most heartfelt and cordial thanks to my friends especially to Perna Tiwari who always stood by my side during all the tough times. My eternal gratitude goes to my gracious and affectionate parents for their never ending support.

Date: 15 July, 2015

Place: Patiala

  
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## **ABSTRACT**

The present study demonstrated the alginate encapsulation and short term storage system for germplasm storage of synthetic seeds, produced using individual protocorm-like-bodies (PLBs) of newly developed F1 hybrid of *Cymbidium elegans* x *C. aloifolium*. The best alginate concentration for encapsulation was found to be 3% (w/v) and 100 mM calcium chloride solution resulted in maximum response (93.3%) on Murashige and Skoog medium supplemented with 2.5 µM BAP (6-Benzylaminopurine). The encapsulated PLBs showed different response after storage at different temperature. The multiplication frequency declined on both storage temperatures tested i.e. 4°C and 25°C. Encapsulated PLBs stored at 4°C showed survival for longer storage while capsules stored at 25°C resulted in rapid deterioration within 90 days. This indicates that storage at low temperature is more effective for synthetic seed of this new F1 hybrid. Random amplified polymorphic DNA (RAPD) and Inter simple sequence repeat (ISSR) markers were used to test the clonal fidelity of plantlets emerged from encapsulated beads stored at 4°C to study post storage genetic stability. Results of molecular markers showed absence of any polymorphic band, thus multiplied encapsulated beads stored at 4°C to study post storage genetic stability. Results of molecular markers showed absence of any polymorphic band, thus multiplied encapsulated PLBs were found true to type.

**Keywords-** Germplasm conservation, Clonal fidelity, synthetic seeds, Protocorm-like-bodies, Storage.

**Abbreviations- PLB-** Protocorm-like-bodies,

**MS-** Murashige and Skoog,

**BAP-** 6-benzylaminopurine

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## **LIST OF ABBREVIATIONS**

%	Percent
g	Gram
mg	Miligram
ng	Nano gram
mM	Milli molar
nmol	Nano mole
μl	Micro litre
CTAB	Cetyltrimethyl Ammonium Bromide
EDTA	Ethylene Diamine tetraacetic Acid
TE	Tris-EDTA
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide Triphosphate
PCR	Polymerase Chain Reaction
RAPD	Random Amplification of Polymorphic DNA
ISSR	Inter simple sequence repeat
bp	Base Pair
min	Minutes
BAP	6-benzylamino purine
DMRT	Duncan's multiple range test

Orchidaceae, one of the largest and most diverse family of flowering plants, including up to one tenth of all flowering species of the world (Dressler 1993). Orchids comprise a range of flowers varying in size, shape and color. The family represents wide distribution and numerous species, with over 800 genera and 25,000 species (Chugh et al. 2009). Genus *Cymbidium* of Orchidaceae family exhibit 52 species and hundreds of manmade hybrids varieties such as *cymbidium X ballinum*, *cymbidium X florida* etc (Pence 1999). *Cymbidium* plants are epiphytic in nature and mainly found in India at the Khasi Hills of Meghalaya, Sikkim-Himalaya, Arunachal Pradesh and Himachal Pradesh (Rout and Jain 2004).

*Cymbidium* plant has prominent place in floriculture industry due to charismatic esthetic value of its flowers (Saiprasad and Polisetty 2003). The plant is most popular in national as well as international market within wholesale value estimated at \$126 million (Tandon et al. 2009). Apart from esthetic value, *cymbidium* plants are also known for their therapeutic properties (Hossain 2011). These plants are commonly used by tribal people in north-eastern India due to its medicinal properties (Balasubramanian et al. 2000). This plant has high utility in enhancing wound healing ability (Medhi and Chakrabarti 2009). *Cymbidium* seed are used by the tribal people in the treatment of burns and weakness of eyes (Radhika et al. 2013).

Currently, due to high economical demand in floriculture market, orchid plant are collected in a very high number from their natural habitat leading to overexploitation (Kala 2009). For conservation of diverse genetic resources conventional method involves seed storage and storage of parental stock (Ellstrand and Elam 1993).

Under natural conditions the propagation rate of orchid is very low as the seed germination is very poor (0.2-0.3%) only, due to lack of endosperm and heterozygosity of seed (Vij and Pathak 2012). Plant tissue culture through *in vitro* seed germination holds potential to overcome problem of low germination rate associated with conventional mass propagation of members of Orchidaceae.

Micropropagation refers to an area of plant tissue culture for vegetative propagation of crops and ornamental plants. This technique involves multiplication of plant through variety of explants, such as auxiliary buds, apical shoot and meristem culture under specialized physicochemical conditions (Chandra et al. 2010). Micropropagation method has emerged as

an efficient tool to improve horticulture and floriculture plant yield (Standardi and Picconi 1998).

Although, small explant requirement and seasonal independence are some of the advantages associated with micropropagation technique but drawbacks such as unpredictable mutations leading to somaclonal variations attracted research studies to find out alternative methods for germplasm conservation (Khoddamadeh et al. 2011).

One such method is artificial seed technology which have potential to overcome micropropagation based problems (Larkin and Scowcroft 1981). Apart from lesser chances of somaclonal variation, various advantages of synthetic seed technology includes transportation, easy handling, germplasm exchange and long term storage etc. (Rai et al. 2009).

Synthetic seed is referred to as artificially encapsulated embryo or vegetative propagules like auxiliary buds, shoot tips, protocorm like bodies or other tissues. It have potential to grow into whole plant under *in vitro* and *in vivo* conditions, and retains this potential even after storage (Ara et al. 2000).

The principle of encapsulation involves the ion-exchange phenomena between the  $\text{Na}^+$  in sodium alginate and  $\text{Ca}^{2+}$  in calcium chloride solution. Round, firm capsules are formed when sodium alginate droplet containing explant are dropped in to calcium chloride solution. Hence, the concentration of gelling matrix, concentration of calcium chloride and exposure time plays an important role in determining the texture and quality of synthetic seeds. (Sai prasad and Polisetty 2003).

In synthetic seed technology major factor that affects encapsulation, concentration of gelling matrix (Mohanraj et al. 2009). For entrapment purpose, matrix or coating should not have any adverse effect on the embryo or propagule and under both *in vitro* and *ex vitro* conditions matrix, it should allow germination of propagule (Sorvari et al. 1997). There are variety of gelling matrix which are used for encapsulation due to their capacity of formation of beads such as- agar, alginate, carboxy methyl cellulose etc. Among these, sodium alginate is used widely due to its various advantages like formation of a protective layer around somatic embryo (Malabadi et al. 2005), Alginate have moderate viscosity and available at low cost, it quick gelling potential when it comes into contact with calcium chloride.

Based upon the method of production, synthetic seeds are mainly classified into two types-

**1) Encapsulated desiccated-** In this type of encapsulation method, explants are hardened to withstand desiccation before encapsulation. Desiccation tolerance can be achieved by slowly decreasing relative humidity, rapidly unsealing petri dish for overnight to dry and by nutrient deprivation. This type of synthetic seed was first reported in coated somatic embryo of carrot (Janick and Kitto 1986).

**2) Encapsulated hydrated-** In this type of encapsulation method, explants are coated by many coating agents such as sodium alginate, sodium pectate and carboxy methyl cellulose, which are tested as hydrogels. This type of hydrogel encapsulation of somatic embryos was first developed in alfalfa in 1988 ((Redenbaugh et al. 1991).

Many ornamental and commercially important plant species are desiccation sensitive and cannot be stored for a long period of time by conventional method that leads to loss of viability. One of the widely used method for *in vitro* germplasm conservation is slow- growth storage. In this method germplasm of plant is used to store at temperature of around 4°C. At low temperature metabolic activities and cellular divisions of plant cell gets slow. Plant material can be stored for a long period of time without any genetic alteration (Engelmann et al. 2008). Artificial seed have potential for germination after storage. In orchids, synthetic seeds are capable of germination without any loss in viability even after storage for 90 days at 4° C (Mohanraj et al. 2009).

After storage, regenerated plant by synthetic seed technology are susceptible to culture stress that leads to somaclonal variations (Larkin and Scowcroft 1981). In commercially important plants such as Orchids, clonal fidelity is a major concern. Several molecular markers are available to confirm the genetic fidelity of regenerated plants for their molecular characterisation. Among various available molecular markers, random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) has been widely used for detection of somaclonal variations, genetic characterization, germplasm evaluation, elucidation of phylogenetic relationship, construction of genetic linkage maps and to study clonal integrity (Mishra et al.. 2011; Srivastava et al. 2009; Mandal et al. 2000). RAPD and ISSR are widely used because of its cost effectiveness and ease.

The present study was aimed to investigate factors that affects production and storage of synthetic seed in *Cymbidium elegans* X *C. aloifolium* hybrid. Further, the work was also focussed to test the clonal fidelity of the plants regenerated from the synthetic seeds following storage.

Orchid plants are known for their wide range of beautiful flowers and possess high commercial value but they are under threat worldwide due to overexploitation by indiscriminate collection and deforestation (Swarts and Dixon 2009). Some reports suggested that orchid plants are mainly affected by nutrient quality of soil which is declining due to human activities and mining (Ganeshiah et al. 1998; Sosa and Platas 1998). It leads to low seed germination rate of orchids under natural conditions. Plant tissue culture through micropropagation is an efficient method to overcome low germination rate in orchids (Gangaprasad et al. 1999). Various reports suggested that micropropagation of orchids can be achieved using different explants such as leaf segments, nodal segment, shoot apices, protocorm-like- bodies etc.(Arditi 2009; Wimber 1963; Kerbauy 1991).

Seeni and Latha (2000), reported rapid multiplication of *Vanda coerulea* orchid. In this study, it was observed that shoot tips of mature plant differentiated to shoot buds after 12 weeks of culture on medium containing 8.8  $\mu\text{M}$  6-benzylaminopurine (BAP) and 4.4  $\mu\text{M}$   $\alpha$ -naphthaleneacetic acid (NAA). A protocol for the rapid micropropagation of *Dendrobium primulinum* Lindl, was developed through the shoot tips explant. In this study, shoot tips were cultured on solidified Murashige and Skoog (MS) medium alone or supplemented with different combinations of plant growth regulators,  $\alpha$ - naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP). Multiple shoots were observed at different combinations of plant growth regulators (Pant and Thapa 2012). Micropropagation of *Dendrobium* was established using shoot meristem explants on various combinations of auxins (IAA, NAA and IBA) and at different sucrose level. It was observed that rooting was rapid when shoot tips were cultured on sucrose alone (Sharon and Vasundhra 1990). A rapid procedure for clonal propagation of *Anoectochilus formosanus* orchid was established by induction of shoot proliferation from shoot tip explant. In this study, Hyponex media supplemented with thidiazuron (TDZ) induced multiple shoot formation (Ket et al. 2004).

Apart from shoot tips, leaf segments also proved to be good choice in micropropagation of orchids as they are seasonal independent (Churuchill et al. 1973). In orchids, work has been undertaken on large scale propagation of epiphytic orchid, *Acampe praemors* (Roxb.) by using foliar explants. In this study, developmental stage and leaf orientation play an

important role in shoot regeneration. The MS medium supplemented with N- benzyladenine (BA),

kinetin or thidiazuron (TDZ) was reported favorable for shoot elongation and leaf expansion (Nayak et al. 1997). A protocol for rapid micropropagation of *Spathoglottis plicata* orchid was established using leaf explants. In this study, MS medium supplemented with naphthalene acetic acid (NAA) and 6- Benzylaminopurine (BA) proved to be effective for differentiation of PLBs (Teng et al. 1997).

Although, small plant requirement is a major advantage associated with micropropagation but problem of somaclonal variations in regenerated plants attract the researchers to find out alternative methods for conservation of germplasm (Khoddamadeh et al. 2011).

Artificial seed technology is one of the method that may avoid somaclonal variations and prove to be efficient in long term storage (Rai et al. 2009). First time, the production of synthetic seed was developed by encapsulating somatic embryo of carrot followed by dessication (Janick and Kitto 1986). In this approach, various compounds were tested for encapsulation. Among them, polyoxyethylene was selected on the basis of various parameters such as non-toxicity and solubility etc. The major drawback of this method was that the encapsulation process was only possible for desiccation sensitive plants. Later on, a procedure for encapsulation of desiccation sensitive plants by testing a number of hydrogel coating matrix such as carragenan, sodium pectate and alginate were developed (Redenbaugh et al. 1991). According to this report, alginate was found to be more appropriate for encapsulation of somatic embryo of alfa alfa, due to its nontoxic and coating properties. Since, then different plant species have be tested for production of synthetic seeds including ornamental plants, fruits and cereals etc. (Ganapathi et al. 1992; Mandal et al. 2000; Rai et al. 2009).

In encapsulation process, type of explant have been reported to play an important role in synthetic seed formation. Earlier, somatic embryo were widely used as an explant. In recent years, so many reports suggested the possibility of other propagules as an explant alternative such as nodal segments, auxillary buds, protocorm- like bodies etc. (Nhut et al. 2005; Rihan et al. 2011).

In orchids, most widely used explant for synthetic seed production are protocorm-like-bodies which are equivalent to somatic embryos (Reddy et al. 2012). Corrie and Tandon (1993), reported that protocorm-like-bodies of *C. giganteum* converted in to platelets when they were transferred either on sterile soil or nutrient media. They found that encapsulated protocorms like bodies showed conversion frequency of 100% and 84% under *in vitro* and *in vivo* conditions respectively. These studies highlighted the possible for transplant of those propagules directly to soil giving an advantage of easy acclimatization.

Another study on synthetic seed production of medicinal important orchid *Dendrobium nobile* was carried out by encapsulating the protocorm-like-bodies and it was observed that encapsulated PLBs showed higher conversion rate on MS medium (Mohanty et al. 2013). Gantait and Sinnah (2013), reported that alginate encapsulation of protocorm-like-bodies of *Aranda Wan Chark Kuan 'Blue' X Vanda Coerulea* Griffth ex. Lindl, resulted in higher percentage of germination and conversion on MS medium. According to this study, regenerated plantlets from capsules showed higher survival rate.

In encapsulation process, coating matrix associated with plant material plays important role to achieve higher conversion frequency. Earlier reports suggested that majority of spherical beads were obtained with 3% sodium alginate and 100 mM calcium chloride solution (Nayak et al. 1997; Faisal et al. 2012). The regeneration potential of encapsulated explant is highly influenced by coating matrix (Cheruvathur et al. 2013).

Gaintait et al. (2012) reported the alginate encapsulation of protocorm like bodies of *Aranda Wan Chark Kaun 'Blue' X Vanda Coerulea* orchid, by using different concentrations of sodium alginate (1-4%) in complexing with 75 mM calcium chloride. In this study, it was found that superior coating was observed by using 3% sodium alginate with 75 mM calcium chloride resulting in higher conversion frequency. In another report encapsulation of protocorm-like-bodies of *Coelogyna previscapa* Lindl, was carried out by using 3% sodium alginate with 75 mM calcium chloride that resulted in maximum conversion of synthetic seed and emergence of shoot when supplemented on MS medium (Mohanraj et al. 2009). Few reports suggested that lower concentration of sodium alginate (1-2%) were responsible for asymmetric and fragile beads. On the other hand very hard capsules were produced when high concentration of sodium alginate (4%) was used (Ray and Bhattacharya 2010; Singh et al. 2010). Work was also carried out to study the effect of exposure time of calcium chloride

on encapsulation. It was observed that exposure time of 30 minutes to calcium chloride solution absorb optimized amount of calcium ions that leads to round and firm beads formation and results in maximum emergence of roots and shoots, whereas exposure time above 30 minutes proved to be detrimental for shoot emergence from beads (Nagesh et al. 2009).

Synthetic seed technology plays an important role in the short term storage for the conservation of germplasm. Therefore, storage temperature and storage days plays an important role in maintaining the viability of encapsulated propagule (Saiprasad and Polisetty 2003; Sharma et al. 2013). Saiprasad and Polisetty (2003) reported that the encapsulated protocorm-like-bodies of *Dendrobium* on MS medium supplemented with 4.44  $\mu$ M BAP resulted in maximum conversion frequency when stored at 4°C storage and retained viability up to 75 days of storage. In another study *Coelogyne breviscapa*, it was suggested that at the storage temperature of 4°C, encapsulated protocorm-like-bodies showed higher conversion on MS medium containing with IAA (Indole acetic acid) in comparison with 25°C storage (Mohanraj et al. 2009). Several reports concluded the fact that storage at 4°C for synthetic seed are more preferable than storage of 25°C (Chand and Singh 2004; Mandal et al. 2000). However, according to Hung and Trueman (2012), storage temperature of 25°C for synthetic seeds of *Corymbia torelliana* x *C. citridora* found more preferable for regeneration. In this study, encapsulated seeds showed more germination efficiency for longer time.

*In vitro* regenerated plant by synthetic seed technology shows somaclonal variations due to various factors such as explant type, culture condition etc. (Williams et al. 1990). Therefore, to establish genetic uniformity of synthetic seed derived plantlet is very important for plant species including orchids. In orchids few reports (Gantait and Sinnah 2013; Faisal et al. 2012), are available for assessment of post storage stability of converted propagule by using PCR based random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR).

Mohanty et al. (2013) reported uniform molecular profile to assess post storage genetic stability of protocorm like bodies stored for 60 days. In this study, 10 primers were successfully generated 39 scorable bands which confirmed genetic fidelity. In another study, Gantait and Sinnah (2013), reported molecular profiles of regenerated plantlets from encapsulated shoot tips of monopodial orchid hybrid 'Aranda Wan Chark 'Blue'X Vanda

*coerula* orchid. According to this study, nine ISSR primers were successfully employed to assess post storage viability of synthetic seeds stored at 4°C and 25°C. By using nine ISSR primers 51 reproducible monomorphic bands revealed no variation that proved the high genetic stability. A work was being carried out to check genetic stability of *Rauwolfia serpentina* plantlets converted from synthetic seed after storage of 4°C and 25°C by using RAPD and ISSR markers (Faisal et al. 2012).

**Plant material**

Newly breed F1 hybrid cultures of *Cymbidium elegans* x *C. aloifolium* were used in this study. The cultures were maintained on Murashige and Skoog medium (Murashige and Skoog 1962) containing 3.0% (w/v) sucrose and 0.7% (w/v) agar (basal MS medium) supplemented with 2.5  $\mu$ M 6-benzylaminopurine (BAP). The protocorm-like-bodies (PLBs) differentiated on this medium in 21 days old cultures were used in this study.

**Chemicals, glassware and plastic ware**

All chemicals used in this study were purchased from HiMedia Laboratories, Mumbai, India. Enzymes, dNTPs required for amplification were procured from Thermo fermentas. Glasswares such as measuring cylinder, conical flask and pasteur pipette etc, were procured from Tarson Ltd. (Mumbai, India). Plant tissue culture was carried out in 300 ml culture bottles (Kasablanka Corporation, Mumbai, India).

**Optimization of encapsulation protocol**

To achieve uniform calcium-alginate encapsulation, protocorm-like bodies (PLB) of 2-3 mm diameter were individually separated from 21 days old culture under aseptic conditions. PLBs were washed with sterile distilled water before encapsulation. PLBs were then suspended in low-viscosity autoclaved sodium alginate (alginic acid sodium salt from brown algae) gelling agent with concentrations of 1, 2, 3, 4% (w/v). The variable concentrations of sodium alginate were tested to check the optimum concentration to achieve uniform, firm capsules. Aliquotes of sodium alginate solution each having one PLB were aseptically taken in 5 mm-diameter Pasteur pipette and dropped into 100 ml of 100 mM (w/v) autoclaved calcium chloride solution, used as a complexing agent. The droplets containing PLBs were kept in calcium chloride solution for 30 minutes of percolating time to achieve polymerization. After achieving encapsulation, calcium chloride was decanted off and capsules were washed thrice with sterile distilled water. The entire process was carried out in aseptic conditions in a laminar flow chamber.

To optimize the suitable concentration of calcium chloride for encapsulation, different concentrations of calcium chloride (50 mM, 75 mM, 100 mM and 125 mM) were used.

Individual PLBs were isolated from 60 days old culture under aseptic conditions. PLBs were then suspended to gelling matrix of autoclaved 3% sodium alginate (alginic acid sodium salt from brown algae). The different concentrations of calcium chloride were tested to achieve uniform encapsulated beads. Aliquots of alginate containing each PLBs were dropped by 5 mm-diameter pasteur pipette and dropped in to four beakers each containing 100 ml volume of four different concentrations of calcium chloride 50mM, 75mM, 100mM, 125mM. The droplets of PLBs were kept in different concentrations of calcium chloride solution with 30 minutes of exposure time. After achieving polymerization encapsulated PLBs were removed from calcium chloride solution and washed thrice with sterilized distilled water under aseptic conditions. Percolating time or exposure of beads to calcium chloride also plays very important role in encapsulation process as it determines the time required for polymerization between sodium and calcium ions. For optimization of percolating time to produce firm, clear and uniform encapsulated beads, PLBs of size 2-3 mm diameter were taken individually from plant culture under aseptic conditions. PLBs were then suspended in to 100 ml of 3% (w/v) autoclaved sodium alginate and after 5 min, aliquotes of PLBs were taken in to 5 mm-diameter pasteur pipette and dropped into 100 ml of 100 mM calcium chloride solution working as a complexing agent. Droplets containing PLBs were kept under different percolating time i.e. 10, 15, 20, 30 minutes in calcium chloride solution. After each percolating time, encapsulated PLBs were removed from calcium chloride followed by washing with distilled water. The beads produced with each of above treatments, were inoculated on medium to see conversion.

### **Storage of encapsulated Protocorm-like-bodies**

To determine conversion percentage and longevity of capsules after short-term storage at cold temperature (4°C) and room temperature (25°C), PLBs were transferred in to sterilized miniature screw-capped polypropylene vials (measuring 5.5 x 1.5 cm). Each vial contained five capsules and this experiment was performed in triplicates. To assess the effect of storage on conversion efficiency of capsules, vials containing encapsulated PLBs were then kept at 4°C and room temperature (25° C) for 15, 30, 45, 60, 75 and 90 days. The longevity of stored

encapsulated PLBs, was assessed by taking vial at the end of each storage period and inoculating beads on basal MS medium supplemented with 2.5  $\mu$ M BAP. Data was recorded by observing growth of regenerated plant, days required for multiplication and viability of encapsulated PLBs.

### **Clonal Fidelity Studies**

The tissue grown from each PLBs after 60 days of storage was maintained separately, DNA was isolated from each line to check the clonal fidelity.

### **Isolation of Genomic DNA**

Genomic DNA was isolated from mother plant and germinated encapsulated growing explant, using the modified CTAB method (Doyle and Doyle 1990). Fresh tissue of 2.0 gm weight of each sample was taken and washed with distilled water. It was dried and grounded into fine powder in liquid nitrogen. Fine powder was immediately transferred in to 50 ml clean, autoclaved polypropylene centrifuge tube. Prewarmed (60°C) CTAB extraction buffer (10.0 ml) was added to each sample to make slurry following the incubation at 60°C for 1h in water bath. Chloroform and isoamyl alcohol (24:1 v/v) was added in equal volume to slurry and mixed for about 3 minutes, followed by centrifugation at 5000 rpm for 10 min. Aqueous phase was removed with the help of wide-bore pipette and transferred to clean tube. If color persists in aqueous phase, chloroform extraction procedure was repeated again. DNA was precipitated with 0.66 volume of cold isopropanol followed by incubation for 1 h at -20°C. The supernatant was discarded after centrifugation (10,000 X g for 15 min.) and the pellet was dissolved in 1 ml TE buffer and transfer to microfuge tube. 2 $\mu$ l of preheated RNase solution (10 mg/ml stock) was added and incubated at 37° C for 1h. Equal volume of phenol and chloroform (1:1 v/v) was added to samples followed by gentle shaking and centrifugation (10000 X g for 10 min). Aqueous layer was retained. In this aqueous layer 0.3 volume of 3 M sodium acetate and 0.6 volume of chilled isopropanol was added and incubated at -20°C for 1h. After incubation, samples were centrifuged (1000 X g for 10 min.). The pellet was retained, dried and dissolve in TE buffer and stored at -20 °C.

## **Electrophoresis of DNA on agarose gel**

Qualitative estimation of DNA was performed through electrophoresis. DNA was checked on 0.8% agarose gel (w/v). Gel was prepared by adding 0.32 g of agarose (Life Technologies India Pvt. Ltd.) in 30 ml of 0.5 X TAE buffer (Tris-Acetate-EDTA) buffer. The agarose gel solution was boiled until clear. The molten agarose was cooled to 45°C and 1µl of ethidium bromide (10 mg/ml) was added and poured in to casting tray inserted with combs. The gel were allowed to solidify at room temperature. After loading DNA sample, electrophoresis was carried out in electrophoresis apparatus (Amersham Bioscience,USA) at 50 volts for one hour and bands were visualized on U.V. transilluminator (Viber Loumart, France).

## **Quantification of DNA**

The extracted DNA samples were quantified by spectrophotometric measurement using Nano Drop 1000 Spectrophotometer (Thermo Scientific, Wilmington DE, USA) at absorbance of  $A_{260}$ . The qualitative estimation of DNA was also done by taking the ratio of absorbance at 280 nm and 260 nm. The  $A_{260}/A_{280}$  ratio should be in between 1.8-2.0 for the quantification of DNA. The ratio greater than 2.0 indicate the contamination of RNA, while ratio less than 1.8 indicate the presence of protein contamination.

## **PCR based markers (RAPD and ISSR)**

The 20 µl volume of polymerase chain reaction (PCR) mixture contain 50 ng of genomic DNA, 1.0 U Taq polymerase, 100 mM dNTPs mixture, 2.0 µl reaction buffer (10X) and 10 nM primer, Milli Q water (Millipore India, Bangalore) was added to make up the final volume of 20 µl. Amplification was performed in thermal cycler model Gene Amp 9700. Amplification cycles comprise of initial denaturation at 94°C for 5 min; 41 cycle of : 94 °C for 60 sec, 36°C (55°C in case of ISSR) for 90 sec and 72 °C for 90 sec; with final extension at 72°C for 5 min. Overall, 4 random amplified polymorphic DNA analysis primers (Table 1) and 4 Inter simple sequence repeat primer were used in PCR amplification (Table 2).

## **Agarose gel electrophoresis**

Amplified DNA products were resolved on 1.2% agarose gel dissolved in 0.5X TAE buffer, stained with ethidium bromide. A constant voltage of 55 V was provided for 4-5 h. Visualization of DNA fragment was performed under UV light. The pattern showing bands were photographed as digital picture using Gel doc system (BioRad).

## **Scoring of amplified DNA fragment**

Products were compared to the standard molecular size of 100 bp DNA ladder. Amplified DNA fragments (bands) were well separated are scored as being present or absent for both RAPD and ISSR primer. Data was scored in binary manner as score of 1 represent presence and 0 represent absence of DNA bands.

## **Statistics and data analysis**

Each experiment was repeated in triplicate and each replicate contain five samples. The data were recorded on the basis of parameters such as multiplication percentage, days for multiplication. These data were analyzed on weekly basis to optimize the favorable alginate concentration, calcium chloride concentration, percolating time, storage days and storage temperature. Collected data was analyzed for variance (ANOVA) by using Costat software (Cohort, USA), means were compared through Duncan's Range test. Graphs were plotted by GraphPad Prism 5 software.

The present study is the first report on synthetic seed production on protocorm-like-bodies of F1 hybrid of *Cymbidium elegans* x *C. aloifolium*. The main objective of current study was to evaluate multiplication potential of encapsulated PLBs as a method for conservation of this hybrid.

**1) Effect of different concentrations of sodium alginate on morphology of encapsulated protocorm-like bodies-**

In present study, encapsulated protocorm-like-bodies showed divergence in morphology at different concentrations of sodium alginate (1-4%) with 100 mM calcium chloride solution (Table 1). It was found that, at lower level of sodium alginate (1%), encapsulation of PLBs was very poor and no PLB was coated properly (Figure 1, A). On the other hand, beads which were formed by using 2% sodium alginate possess solid texture and cluster formation, which were too soft for handling (Figure 1, B). The best and most uniform size of beads were found with gelling matrix of 3% sodium alginate. At this concentration beads exhibit round, firm and uniform size and provide ease of handling (Figure 1, C). At higher level of sodium alginate (4%) encapsulated PLBs showed rigidity and presence of short tail (Figure 1, D).

**Table 1- Effect of sodium alginate concentrations on morphology of encapsulated PLBs.**

Different concentrations of sodium-alginate (%) along with 100mM calcium chloride	Texture	Morphology of encapsulated protocorm- like-bodies
1	++	Poor encapsulation
2	++	Solid texture and cluster formation
3	++++	Firm, round and uniform size
4	+++	Rigid, firm and isodiametric

++, Poor quality; +++, Best quality; ++++, Good but very hard.

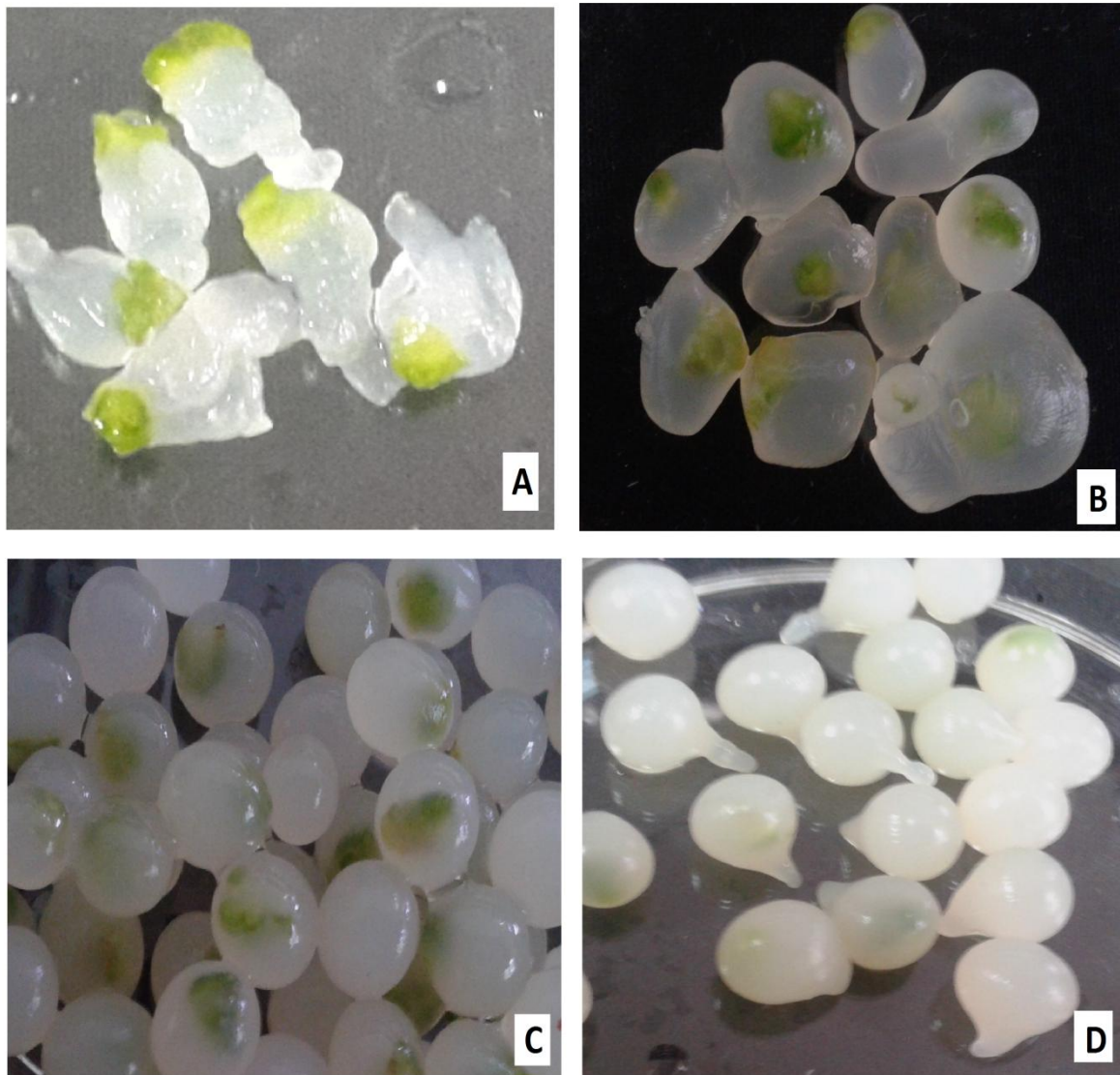
## 2)Effect of different sodium alginate concentrations on frequency of multiplication of encapsulated Protocorm-like-bodies

Further, experiment was carried out to evaluate the effect of different concentrations (1-4%) of sodium alginate on multiplication frequency of encapsulated PLBs. It was observed that PLBs which were formed by using different concentrations of sodium alginate (1-4%) exhibited variable percentage of multiplication and different time requirement for regeneration on MS media supplemented with 2.5  $\mu$ M BAP (6-Benzylaminopurine) (Table 2). At lower concentration of sodium alginate (1%), encapsulation was not suitable because beads were formed of irregular shape, resulting in lower frequency of multiplication (Figure 1, A). At higher concentration of sodium alginate (4%), beads were hard and suppress the emergence of PLBs from coating (Figure 1, D). Thereby, percentage multiplication frequency was decreased up to lower level of 20. In the present study, it was observed that PLBs coated with 3% sodium alginate in combination with 100 mM calcium chloride was found optimal for multiplication of PLBs with high percentage multiplication frequency of 93.3.

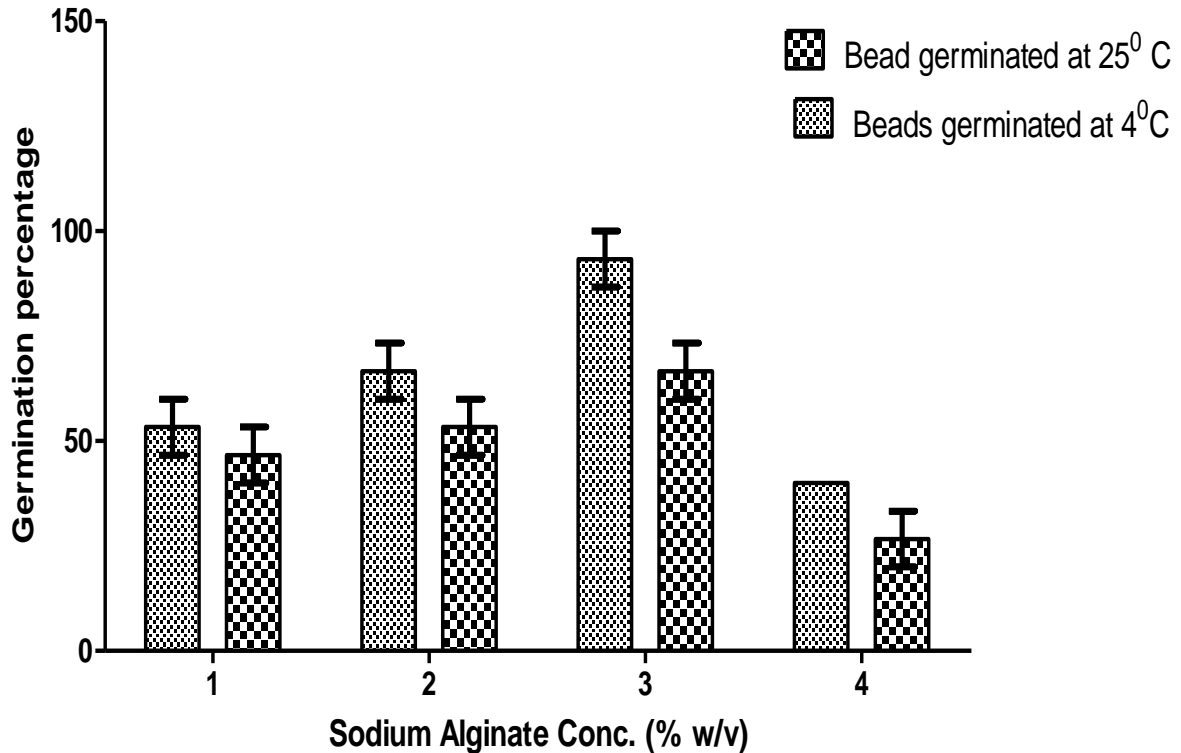
**Table 2: Effect of different concentrations (1-4%) of sodium alginate on percent multiplication frequency of encapsulated PLBs, stored at two different temperatures (4°C and 25°C) for the period of 15 days.**

<b>Combinations of different concentrations of sodium alginate (%) (w/v) with 100 mM calcium chloride</b>	<b>Mean % frequency of multiplication of artificial seeds stored at 4°C, 15 days</b>	<b>Mean % frequency of multiplication of artificial seeds stored at 25°C, 15 days</b>
<b>1</b>	53.33bc	46.66 ab
<b>2</b>	66.66b	53.33 a
<b>3</b>	93.33 a	66.66 a
<b>4</b>	40 c	26.66 b

Data were analyzed by ANOVA and means were compared with DMRT. Values followed by same lower case letter are non-significant at  $P < 0.05$ .



**Figure 1**-Alginate encapsulation of protocorm-like-bodies of F1 hybrid of *Cymbidium elegans* x *C. aloifolium*, by using 100 mM calcium chloride in combination with different sodium alginate concentration (w/v) **A**- 1%, **B**- 2%, **C**- 3%, **D**- 4%

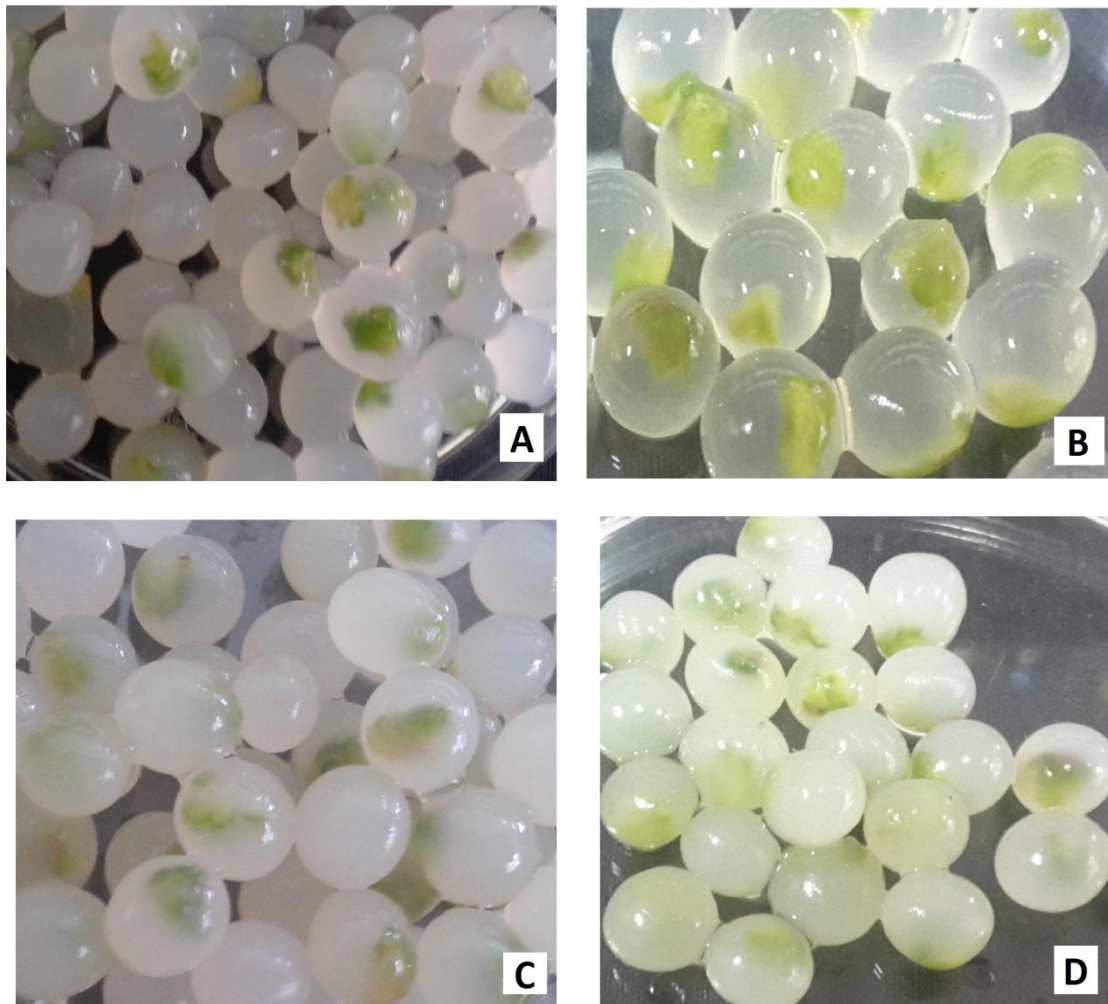


**Figure 2**– Percentage Multiplication frequency of encapsulated PLBs in relation to different concentrations of sodium alginate (1-4%) in combination with 100 mM calcium chloride. Data were recorded after 15 days of storage at two temperatures (4°C and 25°C).

### 3) Influence of different concentrations of calcium chloride on encapsulation and multiplication of PLBs.

Concentration of calcium chloride played an important role on morphology of encapsulated PLBs (Figure 3). From this study, it was observed that the beads obtained from 3% sodium alginate and different concentrations of calcium chloride (50-125mM) exhibit different frequency of multiplication and days required for regeneration (Table 3). Lower level of calcium chloride (50mM) concentration, was not suitable for encapsulation process because beads formed were very fragile and not suitable for handling and resulted in low multiplication percentage (40%) (Figure 3, A). Beads that were formed by using 75 mM calcium chloride solution formed cluster with each other and possess short tail on their surface (Figure 3, B). On the other hand, best beads were obtained by using 100 mM calcium chloride solution (Figure 3, C). It also resulted maximum multiplication percentage of

(93.33%) by using 100 mM calcium chloride. Whereas, beads formed at higher level of calcium chloride (125 mM), resulted in low multiplication percentage of 26.66% (Figure 3,4).

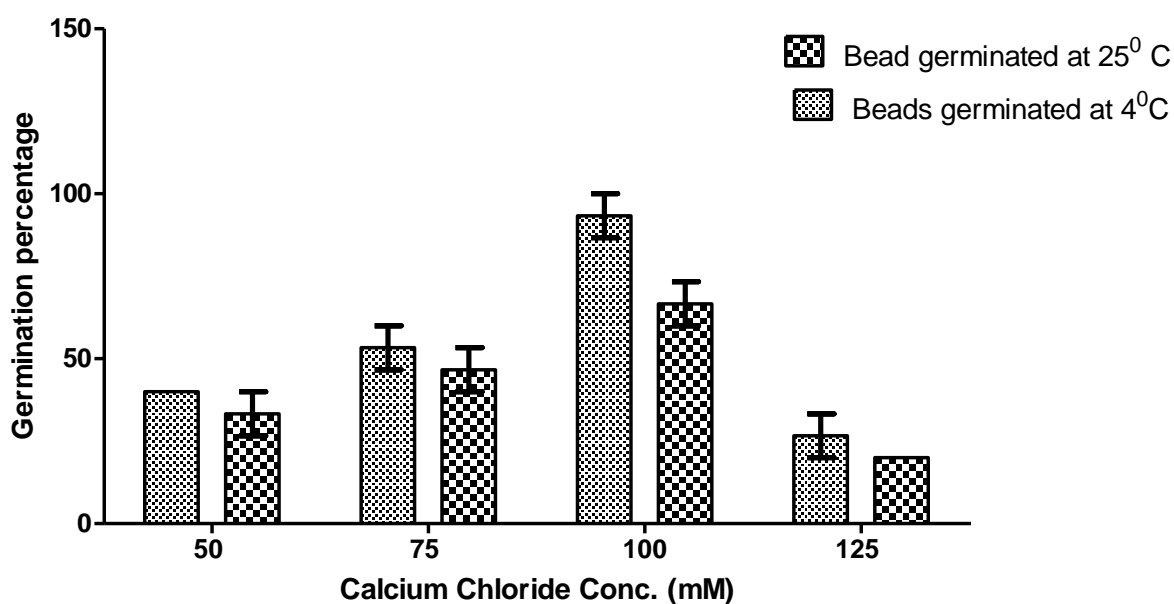


**Figure 3-** Alginate encapsulation of protocorm-like-bodies of F1 hybrid of *Cymbidium elegans* x *C. aloifolium*, by using 3% sodium alginate in combination with different concentrations of calcium chloride. **A-** 50 mM, **B-** 75 mM, **C-** 100 mM, **D-** 125 mM.

**Table 3- Effect of various concentrations of calcium chloride on percentage multiplication frequency of encapsulated PLBs stored at two different temperatures (4°C and 25°C) for a period of 15 days.**

<b>Combinations of different concentrations of calcium chloride (mM) with 3% (w/v) sodium alginate</b>	<b>Mean % frequency of multiplication of artificial seeds stored at 4°C, 15 days</b>	<b>Mean % frequency of multiplication of artificial seeds stored at 25°C, 15 days</b>
<b>50</b>	40 bc	33.33 bc
<b>75</b>	53.33 b	46.66 b
<b>100</b>	93.33 a	66.66 a
<b>125</b>	26.66 c	20 c

Data were analyzed by ANOVA and means were compared with DMRT. Values followed by same lower case letter are non-significant at P<0.05



**Figure 4-** percentage multiplication frequency of encapsulated PLBs in relation to different concentrations of calcium chloride solution (50-125 mM) in combination with 3% sodium alginate. Data were recorded after 15 days of storage at two different temperatures (4°C and 25°C).

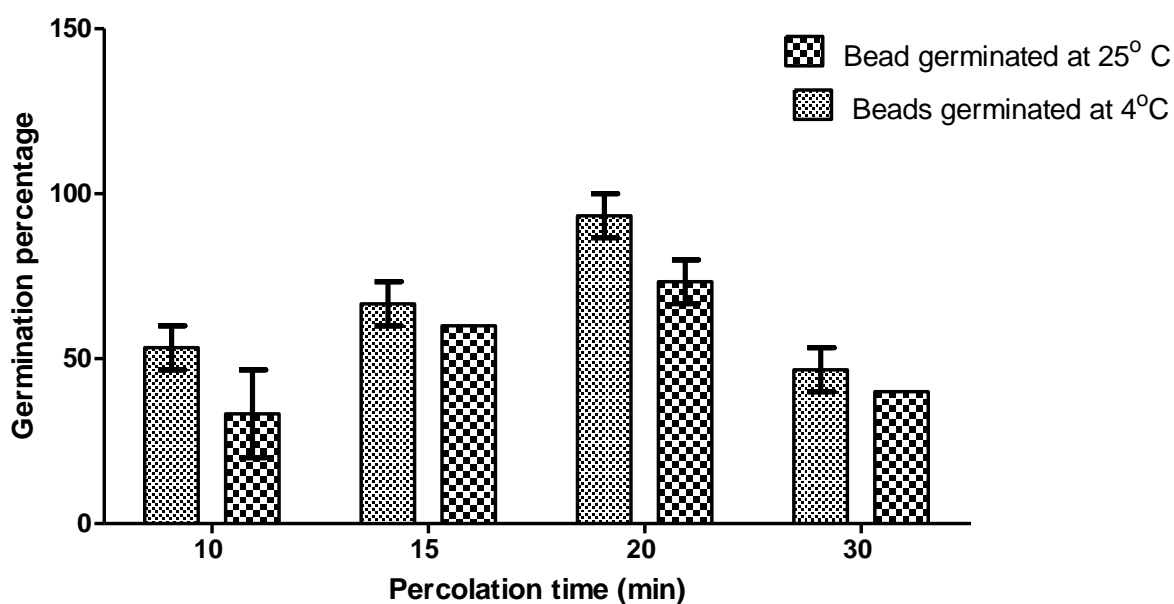
**4) Influence of different exposure time to calcium chloride on percent multiplication frequency of PLBs stored at two different temperatures (4°C and 25°C) for a period of 15 days.**

Further, experiment was carried out to evaluate the effect of different duration of exposure (10min, 15min, 20min and 30 min) to calcium chloride on multiplication of frequency (Table 4). It was observed that exposure time of 20 minutes in calcium chloride was found best for the optimum hardening of beads resulting in higher multiplication percentage of 93.3%. On the other hand, multiplication percentage was found minimum of 53.3%, when the exposure time was less i.e. 10 minutes (Figure 5). Exposure time beyond 30 minutes was found detrimental for multiplication of PLBs.

**Table 4: Effect of different exposure time (10-30 minutes) to calcium chloride on percentage multiplication frequency of encapsulated PLBs stored at two different temperatures (4°C and 25°C) for a period of 15 days.**

<b>Combinations of different concentrations of exposure time (minutes) with calcium chloride (100 mM) and 3% sodium alginate</b>	<b>Mean % frequency of multiplication of artificial seeds stored at 4°C, 15 days</b>	<b>Mean % frequency of multiplication of artificial seeds stored at 25°C, 15 days</b>
<b>10</b>	53.33 b	33..33 c
<b>15</b>	66.66 b	60 ab
<b>20</b>	93.33 a	73.33 a
<b>30</b>	46.66 b	40 b

Data were analyzed by ANOVA and mean were compared with DMRT. Values followed by same case letter are non-significant at P<0.05.



**Figure 5-** Percentage multiplication frequency of encapsulated PLBs in relation with different exposure time to calcium chloride in combination with 3% sodium alginate and 100 mM calcium chloride. Data were recorded after 15 days of storage at two different temperatures (4°C and 25°C)

### 5) Influence of storage days and storage temperature on percentage multiplication frequency of encapsulated PLBs.

From above observations, sodium alginate (3%) and calcium chloride (100mM) were found best for the ideal bead formation. To assess post storage longevity of encapsulated PLBs, beads were stored at different temperature (4°C and 25°C) for different storage period (Figure 6). The present study revealed that encapsulated PLBs stored at 4°C temperature proved to have more multiplication efficiency in comparison with storage at 25°C (Table 5, Figure 7), Multiplication of encapsulated PLBs were initiated after 8 days of culture on MS medium supplemented with 2.5 µM BAP. (Figure 7) compares the multiplication frequency of encapsulated PLBs stored at 4°C and 25°C for 15, 30, 45, 60, 75 and 90 days of storage. It was observed that, at both storage temperature, multiplication frequency of beads were decreased with increase in storage duration. Encapsulated PLBs stored at 4°C were found to retain viability for longer period in comparison with those stored at 25°C. Encapsulated PLBs stored at 4°C were observed to have higher multiplication potential (40%) even after storage of 60 days and contrastingly, at 25°C storage temperature, encapsulated PLBs were observed

to be shrunken, necrotic and brown in nature. These beads were observed to have percentage multiplication frequency of 33 after 60 days of storages and resulted in complete death with in storage of 100 days.

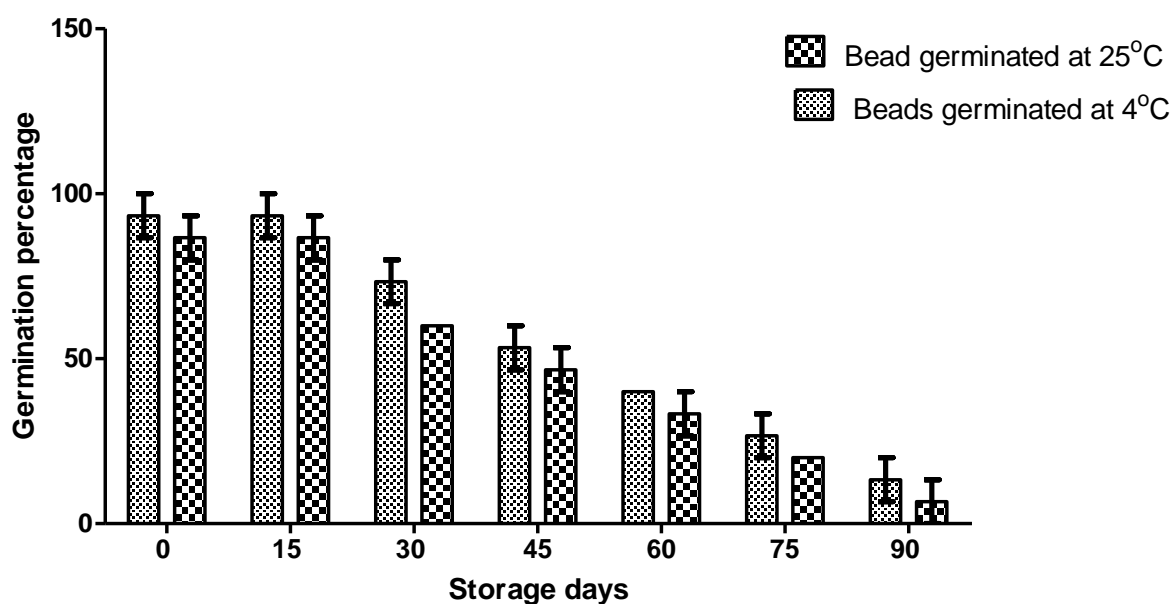


**Figure 6-** Source of explants and multiplication of encapsulated PLBs, **A-** 21 days old culture of *Cymbidium elegans X C. aloifolium* used as a source of, **B-**PLBs used for multiplication; **C-**Encapsulated PLBs; **D-**PLBs shown growth on MS medium supplemented with 2.5 $\mu$ M BAP. **E-** Regeneration and growth of encapsulated PLBs after different period of culture.

**Table 5: Influence of storage days and storage temperature on percentage multiplication frequency of encapsulated protocorm-like bodies.**

<b>Storage Duration (in Days)</b>	<b>Multiplication frequency of encapsulated PLBs stored at 4°C storage.</b>	<b>Multiplication frequency of encapsulated PLBs stored at 25°C storage.</b>
<b>15</b>	93.33 a	86.66 a
<b>30</b>	66.66 b	60 b
<b>45</b>	53.33 bc	46.66 bc
<b>60</b>	40 cd	33.33 cd
<b>75</b>	26.66 de	20 de
<b>90</b>	13.33 e	6.66 e

Data were analyzed by ANOVA and means were compared with DMRT. Values followed by same lower case letter are non-significant at P<0.05.



**Figure 7-** Effect of storage duration (0-90 days) and temperatures (4°C and 25°C) on percentage multiplication frequency of encapsulated PLBs.

### **6) Assessment of genetic stability by using RAPD and ISSR markers-**

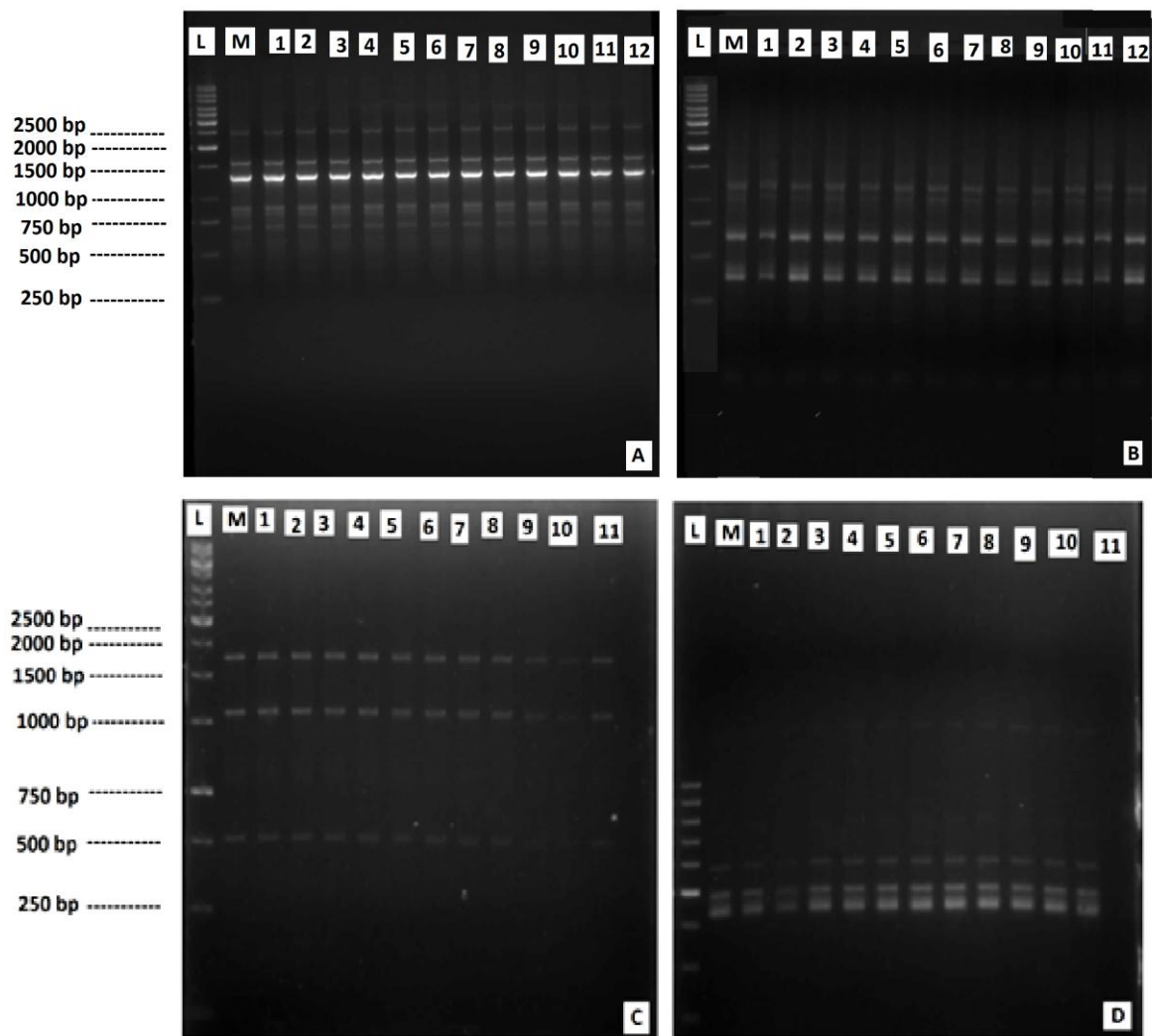
Clonal fidelity of encapsulated PLBs multiplied on MS media supplemented with 2.5 µM BAP after 60 days of storage at 4°C, was evaluated for post storage stability assessment by using RAPD and ISSR markers (Figure 8). Four primers each of RAPD (3, 5, 9,8) and ISSR (3, 2, 5, 9) were used for analysis (Table no. 6 and 7). No polymorphic band was observed in both cases. In case of RAPD total numbers of 16 bands in range of 300-2000 were observed, whereas by using ISSR total numbers of 14 bands in range of 400-2000 were observed (Table no. 6 and 7). There is no polymorphic band was detected and all the observed bands were similar to mother plant. This indicated that there is no change in genetic makeup during storage of synthetic seed derived PLBs and proved clonal fidelity even after 60 days of storage.

**Table 6-** List of random amplified polymorphic DNA (RAPD) primers, used for assessment of clonal fidelity of encapsulated PLBs of F1 hybrid of *Cymbidium elegans* X *C. aloifolium*, their sequence number and range of amplified bands.

<b>Primer Number</b>	<b>Primer Sequence</b>	<b>Number of bands</b>	<b>No. of Polymorphism</b>	<b>Size range (bp)</b>
<b>RAPD 3</b>	AGGGCGTAAG	3	0	400-600
<b>RAPD 5</b>	ACCGCGAAGG	4	0	200-800
<b>RAPD9</b>	TGAGCGGACA	3	0	500-2000

**Table 7-** List of Inter-simple-sequence repeats (ISSR) primers used for assessment of clonal fidelity of encapsulated PLBs of F1 hybrid of *Cymbidium elegans* X *C. aloifolium*, their sequence number and range of amplified bands.

<b>Primer Number</b>	<b>Primer Sequence</b>	<b>Number of bands</b>	<b>No. of Polymorphism</b>	<b>Size range (bp)</b>
<b>ISSR 3</b>	GAGAGAGAGAGAGATC	5	0	600-1500
<b>ISSR 2</b>	GAGAGAGAGAGAGACG	2	0	400-2000
<b>ISSR 5</b>	ACACACACACACAC	4	0	100-1000
<b>ISSR 9</b>	GCGCGCGCGCGCGCT	3	0	500-1200



**Figure 8-** Agarose gel electrophoresis of RAPD and ISSR products of multiplied encapsulated PLBs of F1 hybrid of *Cymbidium elegans* x *C. aloifolium*. **A-D-** Genetic profiles of multiplied encapsulated PLBs and mother plant using **A and B-ISSR, C and D-RAPD**, **L-Ladder, M-Mother plant, 1-12- Multiplied PLBs**

In present study, an attempt has been made to produce synthetic seeds by encapsulating protocorm-like-bodies of F1 hybrid of *Cymbidium elegans* X *C. aloifolium* and to study the post storage conversion and assessment of clonal fidelity has been made to find potential of germplasm conservation of this newly developed hybrid. Encapsulation of various explants and their subsequent conversion into plantlets have been reported in many plant species (Ghosh and Sen 1994; Ganapathi et al.1992). However, only few reports are present on propagation of orchids by using synthetic seed technology (Gantait and Sinnah 2012).

In present study, it was observed that, lower concentration of sodium alginate (1-2%) do not coat protocorm-like-bodies properly and resulted in very soft and fragile beads. At higher concentration of sodium alginate (4%) beads formed were hard in nature and affect the regeneration potential by suppressing the emergence of coated PLBs. Therefore, 3% (w/v) sodium alginate was found most effective for synthetic seed formation of F1 hybrid of *Cymbidium elegans* x *C. aloifolium*. Similar observations were made by (Mohanraj et al. 2009) for encapsulation of PLBs in *Coelogyne breviscapa*. In this study, higher germination percentage (95%) of encapsulated PLBs was achieved after 45 days of culture on MS medium.

Concentration of calcium chloride played an important role in multiplication of encapsulated PLBs. In present study, it was observed that at low concentration of calcium chloride (50 mM), PLBs do not coated properly and leads to lower level of multiplication (40%). Whereas, higher concentration of calcium chloride leads to over hardening of encapsulated PLBs that also resulted in lower multiplication frequency of 26.66%. Mohanty and Das (2013), reported the simiar study on encapsulation of *Dendrobium densilorum* by using PLBs. It was observed that higher germination frequency was achieved by using 100 mM concentration of calcium chloride.

Multiplication frequency of encapsulated PLBs was also influenced by exposure time in calcium chloride. In this study, higher multiplication frequency of 93.33% was observed in 20 minutes of exposure time to calcium chloride. Whereas, exposure time beyond 30 minutes proved to be detrimental for multiplication of encapsulated protocorm-like-bodies. Saiprasad and Polisetty (2003), reported the similar study in encapsulated PLBs of *Dendrobium*. In this

study higher germination percentage was observed by using 20 minutes of exposure time to calcium chloride.

The one of the main feature of encapsulated protocorm-like bodies is that they retain viability even after storage at different temperature and storage days. Similar reports on storage of synthetic seed at 4°C in comparison with 25°C was made in *Coerulea* orchid (Gantait and Sinnah 2013). Beads stored at 4°C were found to show longer viability, whereas, beads stored at 25°C showed lower regeneration potential. This indicated that synthetic seed stored at 4°C were having higher multiplication potential even after 60 days of storage as compared to beads stored at room temperature. The higher regeneration potential could be due to matrix that not only coat PLBs but also protect the tissue from any injury during desiccation. According to Redenbaugh (1991), beads can potentially serve as a reservoir of nutrient which is important for growth of plantlets.

In this study, clonal fidelity of synthetic seeds stored at 4°C were assessed by through RAPD and ISSR markers. By employing markers no polymorphism was obtained which proved clonal fidelity. Faisal et al. (2012), reported similar study on clonal fidelity of synthetic seed derived propagule of *Ravolfia serpentina* after following storage. In this study plantlets converted from synthetic seed after storage of 4°C and 25°C, were assessed for clonal fidelity by using RAPD and ISSR markers. In this study, polymorphism was detected through 19 RAPD and 7 ISSR primers.

Protocol development for artificial seed production and storage of F1 hybrid of *Cymbidium elegans* x *C. aloifolium* can be useful for production of high quality synthetic seed and can be useful for conservation of germplasm. Present study have a potential to be used as a platform for further conservation studies on other orchid species. To check any kind of somaclonal variation, molecular markers are of widely use.

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