Micropropagation and Synthetic Seed Formation in newly developed interspecific F1 hybrid of *Cymbidium giganteum* × *Cymbidium elegans*

A Dissertation

Submitted in partial fulfillment of the requirement for the award of degree of

Masters of Technology

In

BIOTECHNOLOGY

Under the guidance of

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DECLARATION

I declare that the thesis entitled ***Micropropagation and Synthetic Seed formation in newly developed F1 interspecific hybrid of Cymbidium giganteum× Cymbidium elegans*** is a bona fide work under the supervision and guidance of **Dr. Anil Kumar**, Associate Professor, Department of Biotechnology, Thapar University Patiala.

I also declare that this thesis or any other part of this thesis has never been submitted for any degree in this or any other university.

Sumegha Kohli

Place: Patiala

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CERTIFICATE

It is certified that this thesis entitled ***Micropropagation and Synthetic Seed formation in newly developed F1 interspecific hybrid of Cymbidium giganteum× Cymbidium elegans*** is a bona fide work by **Miss Sumegha Kohli (Roll No. 601404022)** and is being submitted in the partial fulfilment of the requirements for the award of the degree of Masters in Technology in Biotechnology to Thapar University, Patiala. This work has been carried out under my supervision and guidance.

It is also certified that this thesis or any other part of this thesis has never been submitted, neither in part nor in full to this university or any other university for the award of any degree.

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Blessed are those that can

give without remembering

and receive without forgetting

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ABBREVIATIONS

S.No	Abbreviation	Full form
1.	PLBs	Protocorm Like Bodies
2.	PGRs	Plant Growth Regulators
3.	BAP	6-Benzylaminopurine
4.	MS	Murashige and Skoog medium
5.	GA_3	Gibberelic Acid
6.	TDZ	Thidiazuron
7.	EDTA	Ethylene Diamine tetra acetic Acid
8.	NAA	1- naphthalene acetic acid
9.	IAA	Indole Acetic Acid
10.	M	Molar
11.	mM	Millimolar
12.	µM	Micromollar
13.	ºC	Degree Celsius
14.	gm	Gram
15.	L	Litre

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ABSTRACT

Cymbidium (family *Orchidaecae*) is a group of important ornamental plants used as pot plant and cut flower. The present work was focussed to study the micropropagation, maturation of PLBs and synthetic seed formation in newly developed F1 hybrid of *Cymbidium giganteum* × *Cymbidium elegans*. Multiplication of *Cymbidium* cultures was done using PLBs on MS basal media supplemented with 2.5µM BAP. Maturation of PLBs was carried out to achieve their efficient conversion into plantlets. Maturation was attempted on basal MS medium containing different concentration of sucrose or mannitol.

Highest maturation frequency of PLBs was observed on medium containing 261 mM sucrose. PLBs matured on medium containing 261 mM sucrose were used for conversion on MS medium variously supplemented with BAP and GA_3. It was interesting to note that PLBs matured on 261 mM sucrose and 247 mM mannitol concentration showed 81.207% and 78.127% germination respectively after 30 days of culture.

Matured PLBs (on 261 mM sucrose) were encapsulated into sodium alginate gels to produce synthetic. The storage of synthetic seeds was studied at 25ºC and 4ºC. The synthetic seeds stored at 4ºC were viable for longer period as compared to those stored at 25ºC. Higher conversion frequency was also observed in PLBs stored at 4ºC.

CHAPTER 1

INTRODUCTION

Cymbidium (Family *Orchidaecae)* also known as boat orchid comprises 52 evergreen species forming a cluster of important ornamental plants. This genus of orchid is found in tropical and subtropical regions of Asia which includes northern India, China, Malaysia, Philippines, Borneo and northern Australia. The flowers are beautiful, have long vase life and come in different colours and architecture (National Research Centre for orchids 2011). Temperature range of 7ºC to 12ºC during night is required for the production of excellent flowers. High temperatures during day time do not seem to hamper their growth as long as temperatures at night are within the range. *Cymbidiums* need to be protected from direct sunlight and should be kept in filtered light (Haworth 2011). *Cymbidiums* are a great choice for celebrations and weddings. These are used as ornamental plants, cut flowers and potted plants. *Cymbidiums* are million dollar industry as cut flowers and fetch high price in the national as well as international market. Approximately 8% of world's floriculture trade centres around *Cymbidiums*.

Fig 1:A. *Cymbidium giganteum* B-C *Cymbidium elegans*

Classification:

Kingdom *Plantae* – Plants

Subkingdom *Tracheobionta* –Vascular Plants

Superdivision *Spermatophyta* – Seed plants

Division *Magnoliophyta* - Flowering Plants

Class *Liliopsida* - Monocotyledons

Subclass - *Liliidae*

Order – Orchidales

Family *Orchidaecae* – Orchid family

Genus- *Cymbidium*

Cymbidiums are outbreeders and can be obtained by interspecific hybridization. First generation interspecific hybrid of *Cymbidium* was developed between *Cymbidium lowianum* × *Cymbidium tigrinum* (Devdas et al. 2012).

Orchids multiplication through seeds is at low rate (Tandon 2003) i.e. orchids require an association with fungal partner (mycorrhiza) for its seed germination and growth because seeds are undeveloped seeds that lack food and nutritional reserves and embryo are under developed so they depend on mycorrhizae for their germination and growth (Arditti 1967).

Difficulty in the seed germination in the orchids was reported due to certain environmental conditions (Knudson 1922). Knudson studied seed germination in epiphytic (Laelia Catlleya) orchid using mineral media supplemented with various sugars.

From the germinated seeds, Begum et al. (1994) obtained the callus that formed PLBs (Protocorm like bodies) in *Cymbidium ensifolium* var. Misericors

These PLBs are similar to somatic embryos (Silva and Tanaka 2006). These PLBs are formed either by direct embryogenesis (without callus formation) or indirect embryogenesis (with callus formation) (Hossain et al. 2013 and Silva 2013 b).

Very few reports have been focussed on the maturation of PLBs in orchids (Jheng et al. 2006, Tremblay and Tremblay 1995). The effect of carbohydrate sources (sucrose, trehalose and maltose) on the PLB proliferation and development in *Oncidium* from embryogenic cultures and higher concentration of different types of sugars turned green PLBs to yellow and enhanced PLB proliferation (Jheng et al. 2006)

The high sucrose concentration in the medium is better for maturation as it roses the osmotic pressure of medium (Tremblay and Tremblay 1995). High sucrose concentration was successfully used for maturation (Triagno and Gray 2010)

Many workers reported the use of PLBs for synthetic seed formation (Gantait et al. 2012, Mohanty and Das 2013, Bustam et al. 2013). Synthetic seeds are encapsulated units containing vegetative propagules (somatic embryos or PLBs). Synthetic seeds have been widely used for germplasm conservation, maintenance of true breeding lines, easy transport and possess low genetic variation (Ravi and Anand 2012) and have short term storage potential without losing viability.

Gantait et al. (2012) isolated the PLBs from grown cultures of *Avanda* Wan Chark Kuan 'Blue' × *Vanda coeruleae* ex. Lindl. (AV) and encapsulated using 3% sodium alginate as gelling agent and 75 mM calcium chloride as complexing agent.

Mohanty and Das (2013) prepared various concentrations of sodium alginate in the range of 2, 3, 4 and 5 % (w/v) and calcium chloride in the range of 50,100 or 150 mM (w/v) for encapsulation of PLBs of *Dendrobium densiflorum* and found the best encapsulation matrix was obtained using 3% sodium alginate and 100 mM calcium chloride.

To determine the effect of storage on germination potential and conversion, encapsulated explants were stored under different temperatures. Some reports have been focussed on the storage of encapsulated beads. Gantait et al. (2012) performed storage experiment in which the encapsulated beads were kept at two different temperatures at 4ºC (low temperature) and at 25ºC (room temperature) and found that bead germination and conversion were better achieved at room temperature (25ºC).

It takes time for the seeds stored at low temperature to recover from cold stress when they shifted to the conversion medium (Ballester et al. 1997). Many researchers reported the plantlet regeneration from encapsulated PLBs (Naing et al. 2011, Mohanty and Das

2013). Bustam et al. (2013) cultured the encapsulated PLBs of *Dendrobium* 'Shavin White' on semi solid half strength MS basal medium. After 37 days of culture, 76 % encapsulated PLBs converted into complete plantlet.

AIM OF THE PRESENT STUDY

Being an important ornamental plant *Cymbidium* has become the centre of the national as well as international floral market. Even though huge work has been done on the synthetic seed production of other orchids like *Dendrobium densiflorum* (Mohanty and Das, 2012) but very less work has been reported on the *Cymbidium* orchid. The maturation and germination in orchids has been reported in very less number of orchids. Although *Cymbidium* is very important ornamental plant but very few studies has been reported on it. The present study was taken up in the cultures of newly developed F1 hybrids of *Cymbidium giganteum* × *Cymbidium elegans* with the following objectives.

Objectives

1. I*n vitro* multiplication of PLBs of *Cymbidium*
2. Maturation of the PLBs for efficient conversion into plantlets.
3. Encapsulation of mature PLBs and optimization of their storage time vis-a–vis storage temperature.

CHAPTER 2

REVIEW OF LITERATURE

Cymbidiums are considered to be one of the most popular flowering orchid as these can survive in cooler and drier conditions. However, the demand of orchid is relatively higher while its multiplication rate through seeds is slower (Tandon 2003). Therefore, requires a method of fast multiplication such as micropropagation. With increase in the demand of the orchids, there is an increase in the production of native terrestrial orchids. But there is difficulty in the seed germination. Kauth (2006) reported the *in vitro* seed germination in two native terrestrial orchids viz. *Calopogon tuberosus* and *Socoila lanceolata*. Seeds of each of the species were inoculated on three different media combinations and incubated under different light conditions. Seeds of *Socoila lanceolata* showed low germination in all the combinations while the seeds of *Calopogon tuberosus* showed high germination in all the light treatments with higher percent of germination on KC (Knudson C) medium (Knudson 1946).

Shadang et al. (2007) studied seed germination of *Hygrochilus parish* by using different formulations of media. Seeds of this specie were inoculated on eight different types of media combinations viz. MS (Murashige and skoog 1962), MKC (Modified Knudson C) (Knudson 1946), V&W (Vacin and went 1949), I&Y (Ichihashi and Yamashita) (Ichihashi et al. 1998) and their respective half strengths. Best seed germination was observed on half strength MKC.

Orchids usually multiply by producing PLBs which are equivalent to somatic embryos (Silva and Tanaka 2006). Use of various Plant Growth Regulators (PGRs) in proliferation or multiplication of PLBs has already been reported (Naing.et al. 2011). In the above mentioned report, the use of NAA and BAP for PLB proliferation has been beneficial. (Wang and Tian 2014) used the basal surface of protocorms and cut end of shoot tips to induce green calli. Better callus induction was observed from protocorms than shoot tips.

Some scientists reported the maturation of somatic embryos in plants like *Paulownia elongata, Oncidium* (Ipekci and Gozukirmizi 2003, Shadang et al. 2007, Jheng et al.

2006). Jheng et al. (2006) reported the importance of sugars in the formation and development of PLBs in *Oncidium* orchid.

Ipekci and Gozukirmizi (2003) induced the somatic embryogenesis in *Paulownia elongata* using leaf and internode as explants on MS basal medium. The explants with induced globular embryos were transferred to different MS medium variously supplemented with NAA, BAP, IAA and kinetin. Percentage of embryos matured from leaf explants were higher than the number of embryos matured from internodal explants.

Mohanty and Das (2013), Perveen and Anis (2014) and Gantait et al. (2012) reported the production of synthetic seeds using PLBs in orchids like *Dendrobium densiflorum* and *Dendrobium* 'Shavin White'.

Synthetic Seeds are the encapsulating units containing vegetative propagules (PLBs or somatic embryo). These have been widely used for germplasm conservation. In present times seed banks are used for maintaining live plants in fields which is costly and labourous method. Therefore, storing the live plants in the form of artificial seeds will help retaining the clones in a limited space, under optimum conditions and for longer periods. This is highly beneficial method of short term *in vitro* germplasm conservation especially for tropical species where there is lack of proper conservation means. The common example of this system of conservation is *Vitis spp.* (Towill 1998). Also synthetic seeds are used for maintenance of true breeding lines, easy transport and with the advantage of low genetic variation and have short term storage potential without losing viability (Ravi and Anand 2012). These provide large scale propagation method with high volume and easy handling.

Many scientists have focussed on the production of synthetic seeds. Mohanty and Das (2013) reported synthetic seed production by using different concentrations of sodium alginate and calcium chloride in *Dendrodium densiflorum* using PLBs. Best results were obtained when 3% sodium alginate was used as gelling agent and 100 mM $CaCl_2$ as complexing agent.

Gantait et al. (2012) isolated the old proliferating PLBs for synthetic seed production in *Avanda* Wan Chark Kuan 'Blue' × *Vanda coeruleae* ex. Lindl. (AV). The isolated PLBs were then dipped into different concentrations of sodium alginate (1, 2, 3, 4 and 5%) as gelling agent, using Pasteur pipette dropped the individual PLBs into 75 mM $CaCl_2$ and allowed it to polymerize for 20 minutes to form beads.

Ipekci and Gozukirmizi (2003) studied the encapsulation of isolated somatic embryos of *Paulownia elongata* by dipping the somatic embryos into various concentrations of sodium alginate (1, 2.5 and 3%) (w/v) and each somatic embryos were then dropped into different concentrations of calcium chloride (50, 60 and 80 mM) for 10 minutes to obtain seeds. Bustam et al. (2013) studied production of artificial seeds for direct regeneration on different media of *Dendrobium* 'Shavin White' in which PLBs with shoot were encapsulated in 3% sodium alginate and polymerized in 75 mM calcium chloride to produce artificial seeds. These encapsulated PLBs were then cultured on different media combinations.

To determine the effect of storage on germination potential, encapsulated explants were stored under different storage conditions. Some reports have been focussed on the storage of encapsulated beads. Gantait et al. (2012) performed storage experiment at different durations in which the encapsulated beads were kept in dark at 4 ºC and 25 ºC for 30, 60, 90, 120, 150 and 180 days. They also studied the effect of storage temperature on the direct conversion of capsules. It was found that better results of germination and conversion were achieved when stored at room temperature (25 ºC). As the duration of storage increased the germination and conversion of capsules decreased both at 4 ºC and 25 ºC. At 4 ºC most of the capsules turned brown resulted in their complete death. Whereas, at 25 ºC the capsules were green, had good conversion and germination potential. The percentage of capsules germinated in conversion media when stored at a temperature of 25 ºC was more than the capsules stored for the same time duration at 4 ºC.

Many reports have been concentrated on the regeneration of plantlet (conversion) from encapsulated PLBs (Bustam et.al 2013, Mohanty and Das 2013).

Regeneration maintains the genetic homozygosity in the plants and also enables mass propagation of new plant varieties. Mohanty and Das (2013) studied the effect of strength of medium and PGR on the regeneration of plantlets of *Dendrobium densiflorum.* The encapsulated and non encapsulated PLBs were cultured on to the growth regulator free MS full strength medium, half strength MS medium alone or supplemented with BAP. Studies revealed that MS full strength containing BAP gave maximum results for encapsulated and non- encapsulated PLBs with 100% conversion.

Bustam et al. (2013) studied plantlet regeneration from PLBs varying in size in *Dendrobium* 'Shavin White' by categorizing the PLBs into different sizes and found that germination was not influenced by size of the PLBs.

The present study was conducted on newly developed F1 hybrid of *Cymbidium giganteum* × *Cymbidium elegans.*

CHAPTER 3

MATERIALS AND METHODS

Materials:

Glassware and chemicals

The Glassware used for culture work comprised of 250 ml, 500 ml, and 1000 ml borosil flasks along with graduated measuring cylinder, beakers, and a range of pipettes. Before use, glassware were thoroughly washed with detergent laboline, washed under running tap water and were then placed in3 a clean tray and left to dry. All Glassware were purchased from Borosil Glass Works Ltd. (Mumbai, India). 300 ml culture bottles (Kasablanka Corporation, Mumbai India) were used in which all the experiments were conducted. The routinely used chemicals were purchased from Hi-media Laboratories Pvt. Ltd. Mumbai. Growth regulators and other fine chemicals were purchased from Sigma Chemical Co. (St .Louis, USA).

Plant Material

Cultures of interspecific hybrid of *Cymbidium giganteum* × *C. elegans* were taken from the plant tissue culture laboratory, TIFAC-CORE, Thapar University (Patiala) and were maintained on MS medium containing 87 mM sucrose and 0.7% (w/v) agar as gelling agent. These were subcultured on basal MS medium supplemented with 2.5 μM BAP after regular interval of 21 days. Cultures were maintained at 25 ºC in growth room under white fluorescent light (50μ mole m^{-2} sec^{-1}) and photoperiod of 16 hrs light/ 8 hrs dark cycle.

Preparation of stock solutions

Macronutrients

10 X stock of each of macronutrients of MS medium was made using required amount of each salt and volume was made upto 100 ml using distilled water. The detailed concentration of macronutrients is given in Annexure-1

Micronutrients

100 X stock of all micronutrients of MS medium was prepared using required amount of each salt and volume was made up to 100 ml using distilled water. The detailed concentration of micronutrients is given in Annexure-1

Vitamins

1 mg/ml concentration of stock of each of the vitamins was made using required amount of each salt and volume was made up to 50 ml using distilled water. . The detailed concentration of vitamins is given in Annexure-1

Growth Regulators

1. 6 Benzyl amino purine (BAP)

Stock of 2.5 mM BAP in HCl: 56 mg of BAP was dissolved in 500 μl HCL. The volume was made upto 100 ml using distilled water.

2. Gibberellic Acid (GA_3)

Stock of 5 mM GA_3 was prepared by dissolving 173 mg of GA_3 in 50 ml water and volume was made to 100 ml using distilled water.

Media Preparation

Media was prepared using stocks of macronutrients, micronutrients and vitamins as described by Murashige and skoog, 1962 with 87 mM sucrose and 0.7% agar as solidifying agent. The pH was adjusted to 5.8 using 1 N HCl or 1 N NaOH prior to autoclaving. Plant growth regulators were added as per requirement before autoclaving. Medium was autoclaved for 15 minutes at 121ºC temperature and 15 psi pressure and transferred to storage room where they were kept under aseptic conditions till their further use.

Methodology

Multiplication of PLBs

The multiplication of *in vitro* grown cultures of *Cymbidium* was carried out using PLBs as explants. The glass plate was flamed and was kept for 10 minutes for cooling. Using the forecep, holded the explant and extra agar surrounding the explant was removed with the help of scalpel. Explant were cut into small size of 1-2 cm and inoculated on basal MS medium supplemented with 2.5 µM BAP. Neck of the bottles were sealed using cling film and placed in the growth room at 25ºC ±1ºC with controlled temperature and air.

Maturation of PLBs

In this experiment MS basal media was supplemented with different concentration of sucrose i.e. 87 mM, 174 mM, 261 mM and 348 mM. The single and clustered PLBs were excised from growing cultures and were inoculated onto the media comprising various concentrations of sucrose.

In another set of experiment, different concentrations of mannitol i.e. 0 mM, 82.5 mM, 165 mM and 247 mM were added to basal MS medium containing 87 mM sucrose.

Conversion of PLBs

After maturation of PLBs on different sucrose and mannitol concentrations, the matured PLBs were taken and inoculated on MS medium variously supplemented with BA and GA_3 BA (0- 0.1µM) and GA_3 (0- 1µM) for conversion into plantlets.

Preparation of synthetic seeds

PLBs were used as explants for synthetic seed formation. 3% sodium alginate and 125 mM calcium chloride were used for encapsulation purpose (Ranjan 2015). The encapsulation procedure was carried out by mixing the freshly excised PLBs in the sodium alginate solution and using wide bore glass pipette PLBs were dropped into chilled $CaCl_2$ solution. The droplets containing PLBs were kept in $CaCl_2$ solution for 15 minutes. $CaCl_2$ was then decanted off and encapsulated seeds were washed five times with autoclaved sterile distilled water to remove excess of $CaCl_2$.

Storage of Synthetic Seeds

After encapsulation, the synthetic seeds were transferred into empty bottles and were stored at 4ºC (cold temperature) and at 25ºC (room temperature) for different periods of time (10,20,30,40 days) in order to check the effect of storage on viability and germination potential of stored synthetic seeds.

Statistical Analysis: Unless otherwise mentioned, all experiments were conducted in six replicates with 5 propagules in each vessel. The data were scored and analysed by ANOVA. Means were compared by DMRT at $P \leq 0.05$.

CHAPTER 4

RESULTS

Multiplication

Multiplication of *in vitro* grown cultures of newly developed F1 interspecific *Cymbidium* hybrid i.e. *Cymbidium giganteum*× *Cymbidium elegans* was carried out on the MS medium supplemented with 2.5 µM BAP. It took one cycle of period i.e. 21-30 days to multiply the cultures. The PLBs were used as explants for multiplication..

Maturation

Maturation of PLBs was done on MS medium supplemented with different sucrose or mannitol concentrations.

The effect of sucrose on maturation of PLBs (as single piece) was studied in which, the highest (40.96%) of matured PLBs were obtained on MS media supplemented with 261 mM sucrose after 30 and 40 days of culture followed by MS medium supplemented with 174 mM sucrose concentration. Minimum (13%) PLBs matured on MS medium supplemented with 348 mM sucrose after 40 days. No significant difference in maturation was observed when analysis was made after 30 and 40 days of culture (Table 1)

While with clustered PLBs on medium supplemented with different concentrations of sucrose, the highest number of matured PLBs per clump were obtained on MS medium supplemented with 261 mM sucrose after 30 and 40 days followed by maturation done on MS medium supplemented with 174 mM sucrose concentration. Minimum number of matured PLBs per clump was obtained on MS medium supplemented with 87 mM sucrose concentration. (Table 3)

In the experiment with the PLBs as single piece matured on mannitol after 40 days, the highest (40.33%) of matured PLBs were obtained on MS medium supplemented with 247 mM mannitol. (Table 2)

After 40 days, the number of matured PLBs per clump on MS medium supplemented with 247 mM and 165 mM mannitol were same. Least number of matured PLBs per clump was obtained on MS media with no mannitol (Table 4)

Germination of PLBs

The effect of PGRs on conversion of PLBs matured on different sucrose concentration was studied and it has been demonstrated that after 30 days, the highest (81.2%) of shoot germination was obtained in PLBs matured on 87 mM sucrose which were then cultured on MS medium supplemented with GA_3. Decrease in the sucrose concentration, increased the percentage of PLBs converting to shoots in all combinations of PGRs. Highest percent of germination was observed in MS medium supplemented with GA_3 followed by MS medium supplemented with BA+ GA_3 combination (Table 5)

While in clustered PLBs, the highest (74%) was also obtained in PLBs matured on 87 mM sucrose and cultured on MS medium supplemented with GA_3 but no germination was obtained in PLBs matured on 348 mM sucrose and cultured on MS medium variously supplemented with all combinations of PGRs except GA_3 and in PLBs matured on 261 mM sucrose and cultured on MS basal medium (Table 6)

Single PLBs matured on mannitol containing medium showed the highest (78.12%) of germination was obtained on MS medium supplemented BA+ GA_3 combination followed by MS medium supplemented with GA_3 with no mannitol (Table 7)

With clustered PLBs, the highest (82.77%) germination was obtained on MS medium supplemented with GA_3 followed by PLBs matured on 82.5 mM mannitol which were cultured on MS medium supplemented with GA_3. (Table 8)

After 40 days, the highest (86.9%) germination was obtained in PLBs matured on 87 mM sucrose which were cultured on MS medium supplemented with GA_3. Minimum (6.25% and 8.3%) were obtained in PLBs matured on 348 mM sucrose and then cultured on MS medium supplemented with BA+GA_3 and BA respectively. Percent germination was significantly higher in PLBs matured on 174 mM and 261 mM sucrose than to PLBs matured on 348 mM sucrose which were cultured on MS medium supplemented with all combinations of PGRs. No germination was obtained in PLBs matured on 348 mM sucrose which were cultured on MS basal and MS basal supplemented with GA_3 (Table 9)

While with clustered PLBs, the highest (66.19%) germination was obtained in PLBs matured on 87 mM sucrose and cultured on MS medium supplemented with BA+GA_3 combination followed by BA. Percent germination was significantly higher in PLBs matured on 261 mM and 174 mM than on 261 mM sucrose. No germination was obtained in PLBs matured on 348 mM sucrose which were cultured on MS medium supplemented with all combinations of PGRs except GA_3 (Table 10)

In mannitol, the single PLBs matured on showed a highest (75.65 %) of germination on MS medium supplemented with BA+ GA_3 combination followed by basal PGR-free MS medium. Percent germination was significantly higher in PLBs matured on 165 mM mannitol than on 82.5 mM mannitol. Minimum percent germination was obtained in PLBs matured on 247 mM mannitol and cultured on all media combinations (Table 11)

With the clustered PLBs on mannitol medium, the highest (56.28%) of germination was obtained on MS medium supplemented with GA_3 followed by PLBs matured on 82.5 mM mannitol and cultured on basal medium supplemented with GA_3. Least percent germination was observed in PLBs matured on 247 mM mannitol in all combinations of PGRs (Table 12).

Table 1: The effect of sucrose concentration on maturation of Protocorm like bodies (PLBs) after 30 and 40 days of culture. The PLBs were inoculated as single piece.

Sucrose Concentration (mM)	Percent PLBs maturated (Mean± S.E)	
	(30 days)	(40 days)
87	9.830^{bB} ±6.238	23.124 ^{aAB} ± 6.359
174	38.761 ^{aA}± 8.848	30.216 ^{aA} ± 6.440
261	40.969 ^{aA} ± 7.313	40.969 ^{aA}± 10.06
348	15.861 ^{aB}± 2.139	13.554 ^{aB}± 2.793

Each bottle contained 15 PLBs. Data were analysed by ANOVA and means were compared by DMRT at P≤ 0.5. Mean values followed by same lowercase (within rows) or uppercase letters (within columns) are not significant at P≤ 0.5.

Table 2: The effect of mannitol in MS medium containing 87 mM sucrose on maturation of Protocorm like bodies (PLBs) after 30 and 40 days of culture. The PLBs were inoculated as single piece.

Mannitol Concentration (mM)	Percent Maturation on Mannitol (Mean± S.E)	
	(30 days)	(40 days)
0	8.84 ^{bB} ± 2.24	30.59 ^{aAB} ± 3.676
82.5	17.34 ^{aAB} ± 3.21	26.50^{aB} ± 6.242
165	12.65 ^{bB} ± 3.021	25.01^{aB} ± 6.766
247	23.48 ^{bA} ± 4.09	40.33 ^{aA} ± 3.465

Each bottle contained 15 PLBs. Data were analysed by ANOVA and means were compared by DMRT at P≤ 0.5. Mean values followed by same lowercase (within rows) or uppercase letters (within columns) are not significant at P≤0.5.

Table 3: The effect of sucrose concentration on maturation of Protocorm like bodies (PLBs) after 30 and 40 days of culture. The PLBs were inoculated in clusters.

Sucrose concentration (mM)	Maturation after 30 days	Maturation after 40 days
87	+	+
	+	+
	+	+
174	+ +	+ +
	+ +	+ +
	+ + +	+ + +
261	+ +	+ + +
	+ + +	+ + +
	+ + +	+ + +
348	+ + +	+ + +
	+ +	+ +
	+	+

PLBs matured on different sucrose concentration and data was recorded after 30 and 40 days of culture on different sucrose concentrations in MS basal medium

Note: Each '+' indicates about 5 matured PLBs per clump

Table 4: The effect of mannitol in MS medium containing 87 mM sucrose on maturation of Protocorm like bodies (PLBs) after 30 and 40 days of culture. The PLBs were inoculated in clusters.

Mannitol concentration (mM)	Maturation after 30 days	Maturation after 40 days
0	+	+
	+	+
	+	+
82.5	+ +	+ +
	+ +	+ +
	+ +	+ +
165	+ +	+ +
	+ +	+ + +
	+ + +	+ +
247	+ + +	+ + +
	+ +	+ +
	+ + +	+ +

PLBs matured on different mannitol concentration and data was recorded after 30 and 40 days of culture on different mannitol concentrations in MS basal medium

Note: Each '+' indicates about 5 matured PLBs per clump

GERMINATION OF PLBs

Table5: The effect of PGRs in basal medium containing 87 mM sucrose on conversion of PLBs matured on medium containing different sucrose concentrations after 30 days. The PLBs were inoculated as single piece.

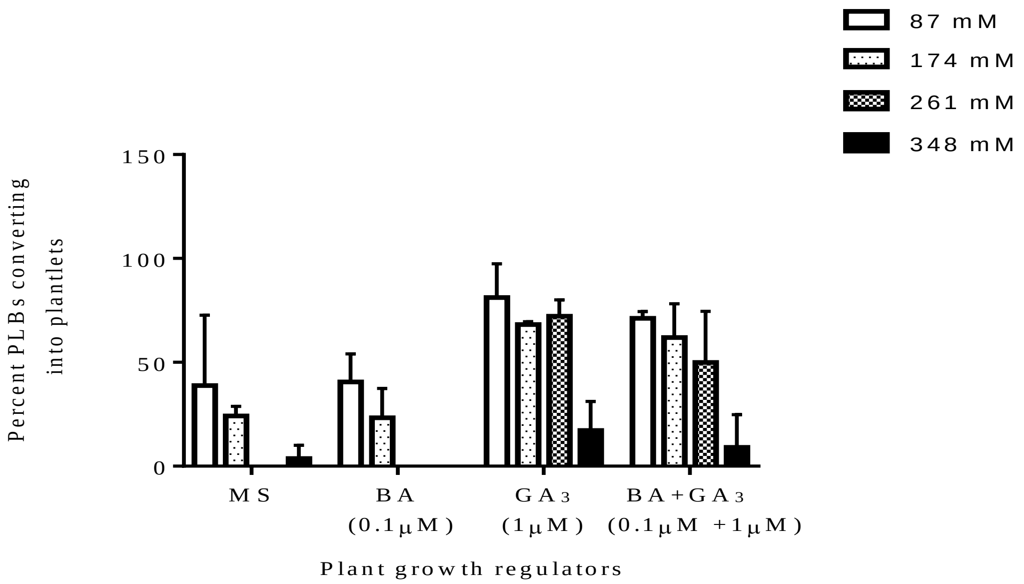
Sucrose Concentration (mM)	Percent PLBs converting into shoots (Mean ±S.E)			
	PGRs			
	BA (0.1μM)	GA_3 (1μM)	MS	BA+GA_3 (0.1μM+1μM)
87	$40.55^{b,A}$ ± 7.83	$81.20^{a,A}$ ± 9.39	$38.86^{b,A}$ ± 19.50	$71.15^{a,bA}$ ±1.92
174	$23.30^{b,A}$ ± 8.16	$68.16^{a,A}$ ± 0.79	$24.22^{b,AB}$ ± 2.66	$61.97^{a,A}$ ± 9.37
261	$00.00^{b,B}$ ± 00.00	$72.22^{a,A}$ ± 4.55	$00.00^{b,B}$ ± 0.00	$49.92^{a,A}$ ±14.20
348	$00.00^{a,B}$ ± 00.00	$17.22^{a,B}$ ± 8.06	$03.70^{a,AB}$ ± 3.70	$09.09^{a,B}$ ± 9.09

PLBs were matured on different sucrose concentration for 30days and data was recorded after 60 days of culture on conversion medium. Data were analysed by ANOVA and means were compared by DMRT at P≤0.5. Mean values followed by same lowercase (within rows) or uppercase (within columns).

Table6: The effect of PGRs in basal medium containing 87 mM sucrose on conversion of PLBs matured on medium containing different sucrose concentrations after 30 days. The PLBs were inoculated in clusters.

Sucrose Concentration (mM)	Percent PLBs converting into shoots (Mean ±S.E)			
	PGRs			
	BA (0.1μM)	GA_3 (1μM)	MS	BA+GA_3 (0.1μM+1μM)
87	$46.06^{b,A}$ ±06.05	$74.00^{a,A}$ ± 03.01	$65.66^{ab,A}$ ±08.01	$50.83^{b,A}$ ±05.42
174	$19.58^{b,B}$ ± 00.42	$37.66^{ab,B}$ ±16.68	$31.51^{ab,B}$ ±11.63	$56.46^{a,A}$ ±09.03
261	$01.93^{b,C}$ ±01.93	$36.52^{a,B}$ ± 10.59	$00.00^{b,C}$ ± 00.00	$41.12^{a,A}$ ± 4.95
348	$00.00^{a,C}$ ±00.00	$03.70^{a,C}$ ± 3.70	$00.00^{a,C}$ ± 00.00	$00.00^{a,B}$ ±00.00

PLBs were matured on different sucrose concentration for 30days and data was recorded after 60 days of culture on conversion medium. Data were analysed by ANOVA and means were compared by DMRT at P≤0.5. Mean values followed by same lowercase (within rows) or uppercase (within columns).



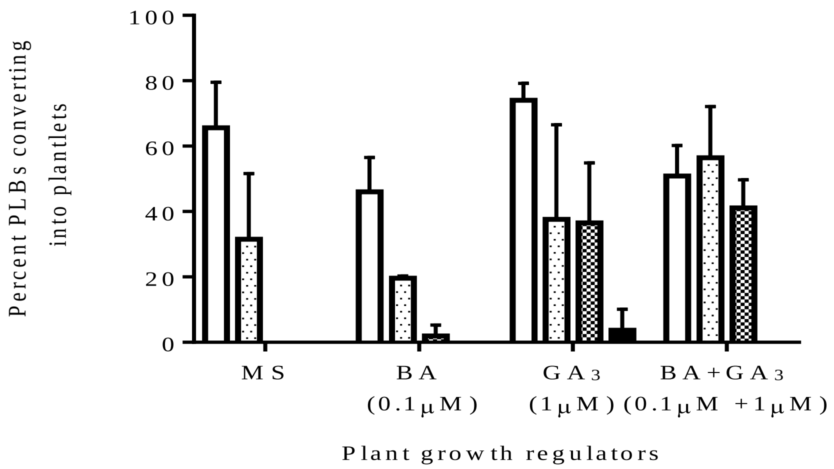


Fig 2. The effect of PGRs on conversion of matured PLBs on different sucrose concentration after 30 days A. Single PLBs B. Clumped PLBs. Lower case letters denote comparison of response of matured PLBs to different PGR combination while uppercase letters represent response of PLBs matured on different sucrose concentrations to one PGR

Table7: The effect of PGRs in basal medium containing 87 mM sucrose on conversion of PLBs matured on medium containing different mannitol concentrations after 30 days. The PLBs were inoculated as single piece.

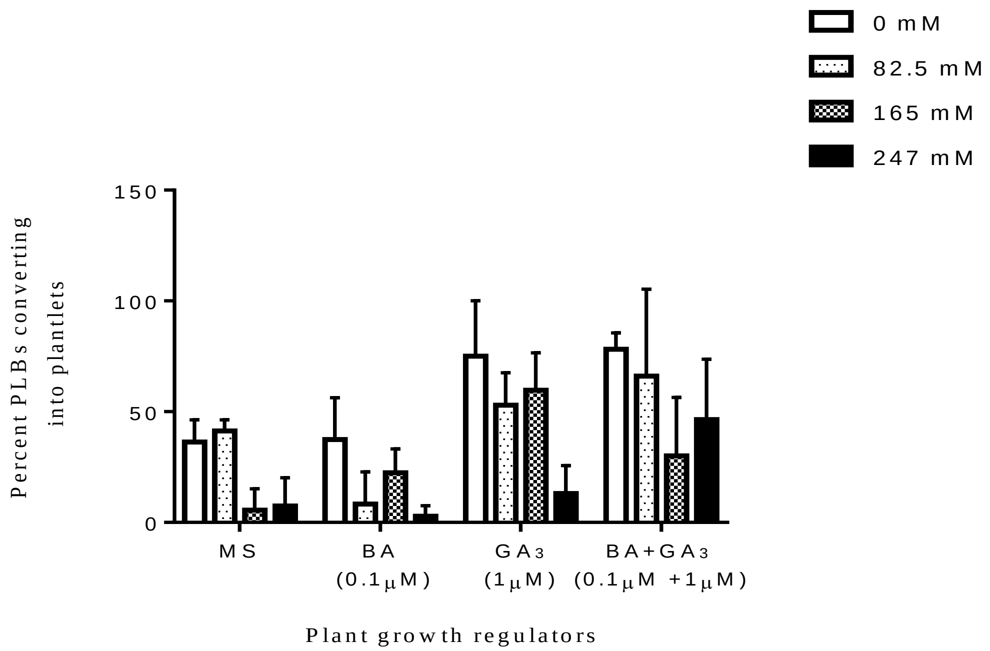
Mannitol Concentration (mM)	Percent PLBs converting into shoots (Mean ±S.E)			
	PGRs			
	BA (0.1μM)	GA_3 (1μM)	MS	BA+GA_3 (0.1μM+1μM)
0	37.38^{bA} ± 10.94	75.00^{aA}± 14.43	36.34^{bA}± 05.78	78.12^{aA}± 04.31
82.5	08.33^{bB} ± 08.33	52.90^{aA}± 08.45	41.32^{abA}±02.86	66.02^{aA}± 22.65
165	22.43^{bAB}±06.22	59.72^{aA}± 09.72	05.55^{bB}± 05.55	30.00^{abB}±15.27
247	02.76 ^{bB} ± 02.76	13.09^{bB}±07.24	07.40^{bB}± 07.40	46.39^{aB} ± 15.74

PLBs were matured on different mannitol concentration for 30days and data was recorded after 60 days of culture on conversion medium. Data were analysed by ANOVA and means were compared by DMRT at P≤0.5. Mean values followed by same lowercase (within rows) or uppercase (within columns).

Table8: The effect of PGRs in basal medium containing 87 mM sucrose on conversion of PLBs matured on medium containing different mannitol concentrations after 30 days. The PLBs were inoculated in clusters.

Mannitol Concentration (mM)	Percent PLBs converting into shoots (Mean ±S.E)			
	PGRs			
	BA (0.1μM)	GA_3 (1μM)	MS	BA+GA_3 (0.1μM+1μM)
0	45.00^{b,A}± 12.58	82.77^{a,A}±07.24	49.09^{b,A}± 03.33	60.74^{ab,A}±06.42
82.5	29.99^{c,AB}±05.25	67.89^{a,A}±09.13	36.66^{bc,A}±06.06	60.59^{ab,A}±08.78
165	04.30^{c,B}± 02.15	32.08^{b,B}±12.08	12.87^{bc,B}±03.70	61.01^{a,A}± 05.65
247	00.00^{c,B} ± 00.00	26.94^{a,B}±01.04	08.30^{b,B} ±05.62	15.68^{a,B}± 15.68

PLBs were matured on different mannitol concentration for 30days were used as explants and data was recorded after 60 days of culture on conversion medium. Data were analysed by ANOVA and means were compared by DMRT at P≤0.5. Mean values followed by same lowercase (within rows) or uppercase (within columns)



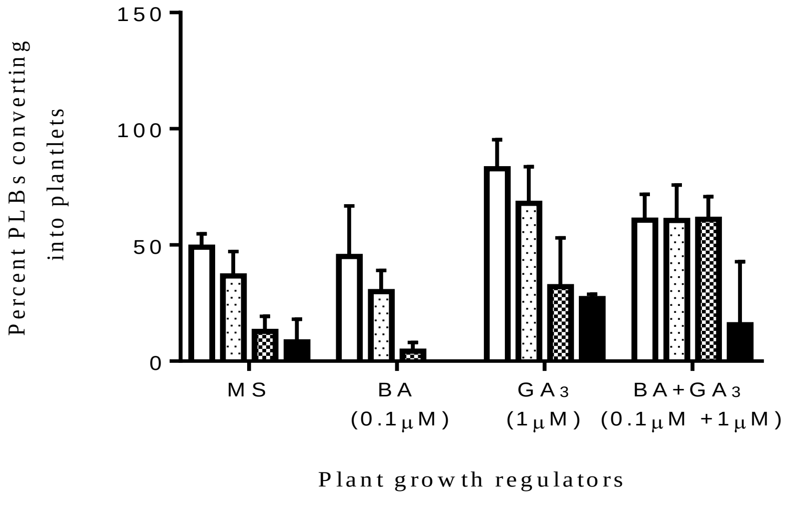


Fig 3. The effect of PGRs on conversion of matured PLBs on different mannitol concentration after 30 days A. Single PLBs B. Clumped PLBs. Lower case letters denote comparison of response of matured PLBs to different PGR combination while uppercase letters represent response of PLBs matured on different sucrose concentrations to one PGR

Table9: The effect of PGRs in basal medium containing 87 mM sucrose on conversion of PLBs matured on medium containing different sucrose concentrations after 40 days. The PLBs were inoculated as single piece.

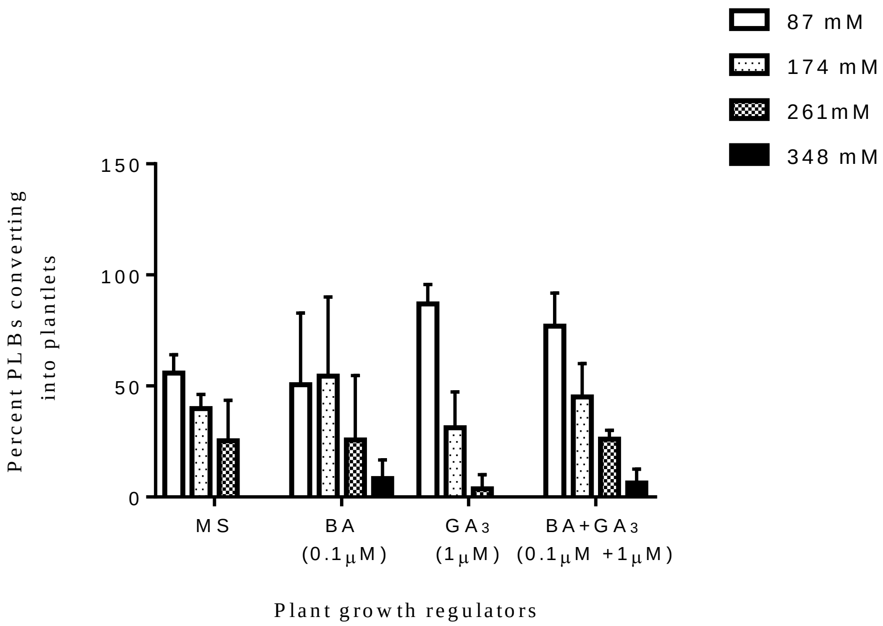
Sucrose Concentration (mM)	Percent PLBs converting into shoots (Mean ±S.E)			
	PGRs			
	BA (0.1µM)	GA_3 (1µM)	MS	BA+GA_3 (0.1µM+1µM)
87	$50.57^{b,A}$± 18.62	$86.93^{a,A}$±05.02	$55.80^{b,A}$± 04.75	$76.93^{a,A}$± 08.50
174	$54.44^{a,A}$± 20.55	$31.22^{a,B}$±09.28	$39.82^{a,AB}$±03.70	$45.00^{a,B}$± 08.66
261	$25.70^{a,AB}$±16.72	$03.70^{b,C}$±03.70	$25.29^{a,B}$± 10.56	$26.11^{a,BC}$±02.24
348	$08.36^{a,B}$ ± 04.80	$00.00^{a,C}$±00.00	$00.00^{a,C}$ ± 00.00	$06.25^{a,C}$± 03.60

PLBs were matured on different sucrose concentration for 40days and data was recorded after 60 days of culture on conversion medium. Data were analysed by ANOVA and means were compared by DMRT at P≤0.5. Mean values followed by same lowercase (within rows) or uppercase (within columns)

Table10: The effect of PGRs in basal medium containing 87 mM sucrose on conversion of PLBs matured on medium containing different sucrose concentrations after 40 days. The PLBs were inoculated in clusters.

Sucrose Concentration (mM)	Percent PLBs converting into shoots (Mean ±S.E)			
	PGRs			
	BA (0.1µM)	GA_3 (1µM)	MS	BA+GA_3 (0.1µM+1µM)
87	$60.23^{a,A}$±05.59	$59.91^{a,A}$± 03.52	$28.56^{b,A}$± 11.16	$66.19^{a,A}$±01.63
174	$00.00^{b,B}$ ±00.00	$40.74^{a,A}$± 11.98	$08.58^{b,AB}$±04.82	$45.20^{a,B}$±00.84
261	$04.76^{c,B}$± 04.76	$29.54^{a,AB}$±17.56	$01.75^{c,B}$ ± 01.75	$12.79^{b,C}$±06.11
348	$00.00^{a,B}$ ±00.00	$03.33^{a,B}$ ± 03.33	$00.00^{a,B}$ ± 00.00	$00.00^{a,D}$±00.00

PLBs were matured on different sucrose concentration for 40days and data was recorded after 60 days of culture on conversion medium. Data were analysed by ANOVA and means were compared by DMRT at P≤0.5. Mean values followed by same lowercase (within rows) or uppercase (within columns).



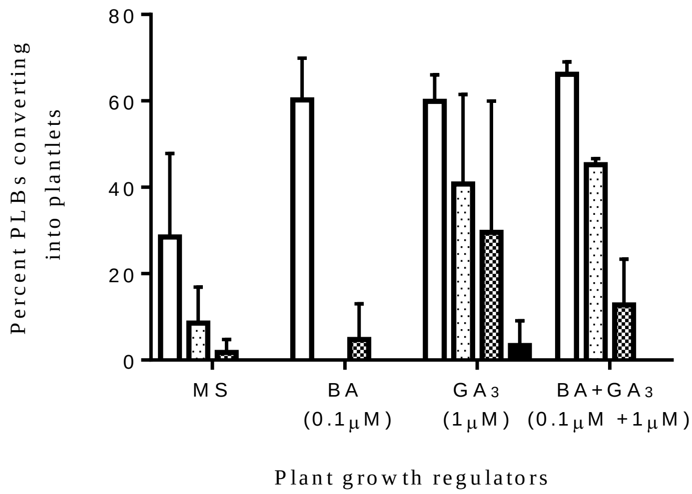


Fig 4. The effect of PGRs on conversion of matured PLBs on different sucrose concentration after 40 days A. Single PLBs B. Clumped PLBs. Lower case letters denote comparison of response of matured PLBs to different PGR combination while uppercase letters represent response of PLBs matured on different sucrose concentrations to one PGR

Table11: The effect of PGRs in basal medium containing 87 mM sucrose on conversion of PLBs matured on medium containing different mannitol concentrations after 40 days. The PLBs were inoculated as single piece.

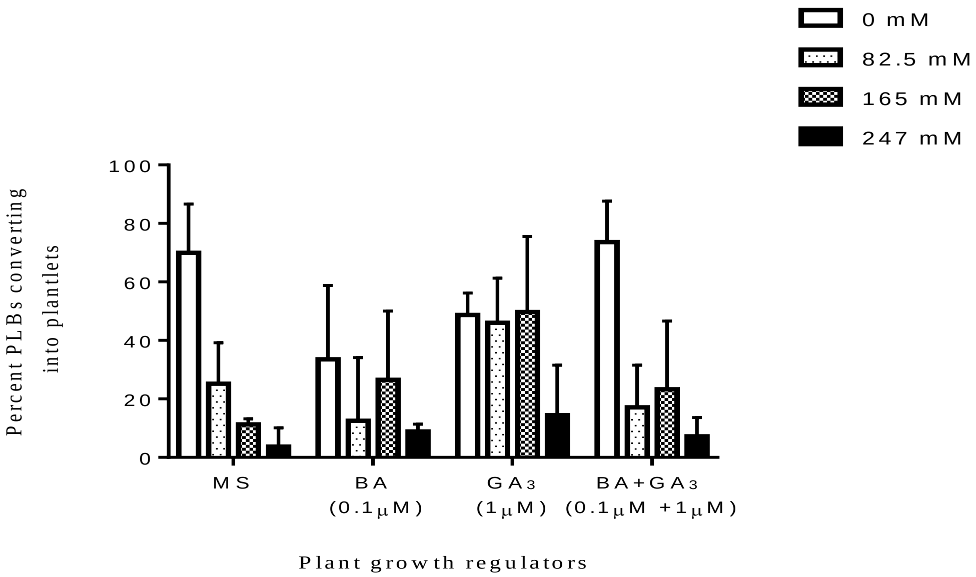
Mannitol Concentration (mM)	Percent PLBs converting into shoots (Mean ±S.E)			
	PGRs			
	BA (0.1μM)	GA_3 (1μM)	MS	BA+GA_3 (0.1μM+1μM)
0	$33.56^{b,A}$± 14.56	$48.71^{ab,AB}$±04.32	$69.99^{a,A}$± 09.62	$75.65^{a,A}$±08.07
82.5	$12.50^{b,B}$ ± 12.50	$46.05^{a,AB}$±08.78	$25.21^{ab,B}$±08.09	$17.12^{ab,B}$±08.34
165	$26.57^{ab,A}$±13.54	$49.72^{a,A}$± 14.90	$11.32^{b,BC}$±01.10	$23.33^{ab,B}$ ±13.47
247	$08.86^{a,B}$±01.45	$14.43^{a,B}$± 09.87	$03.70^{b,C}$±03.70	$07.19^{a,B}$±03.73

PLBs were matured on different mannitol concentration for 40days and data was recorded after 60 days of culture on conversion medium. Data were analysed by ANOVA and means were compared by DMRT at P≤0.5. Mean values followed by same lowercase (within rows) or uppercase (within columns).

Table12: The effect of PGRs in basal medium containing 87 mM sucrose on conversion of PLBs matured on medium containing different different mannitol concentrations after 40 days. The PLBs were inoculated in clusters.

Mannitol Concentration (mM)	Percent PLBs converting into shoots (Mean ±S.E)			
	PGRs			
	BA (0.1μM)	GA_3 (1μM)	MS	BA+GA_3 (0.1μM+1μM)
0	$26.82^{a,A}$± 10.02	$56.28^{a,A}$±12.78	$24.57^{a,A}$± 13.62	$45.90^{a,A}$±05.72
82.5	$18.15^{b,A}$± 05.39	$46.51^{a,A}$±05.52	$23.24^{ab,A}$±05.05	$29.86^{ab,AB}$±12.75
165	$13.01^{ab,A}$±06.51	$32.37^{a,AB}$10.18	$04.76^{b,B}$± 02.38	$19.45^{ab,BC}$±05.65
247	$17.13^{a,A}$±04.49	$05.41^{b,B}$±02.76	$00.00^{b,B}$± 00.00	$02.77^{b,C}$±02.77

PLBs matured on different mannitol concentration for 40days and data was recorded after 60 days of culture on conversion medium. Data were analysed by ANOVA and means were compared by DMRT at P≤0.5. Mean values followed by same lowercase (within rows) or uppercase (within columns)



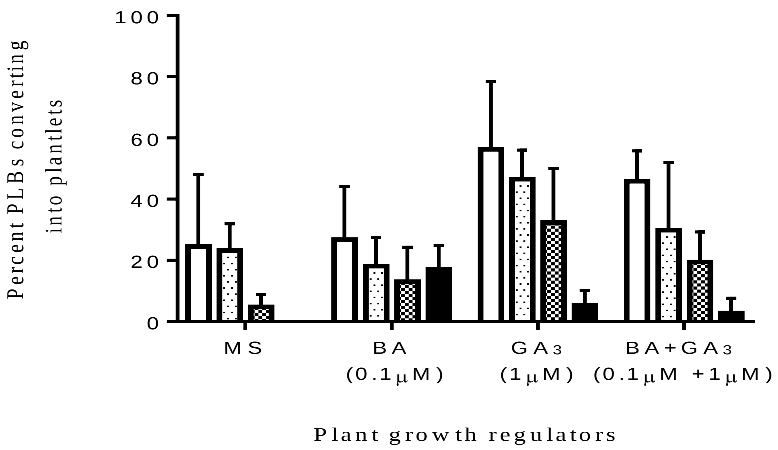


Fig 5. The effect of PGRs on conversion of matured PLBs on different mannitol concentration after 40 days A. Single PLBs B. Clumped PLBs. Lower case letters denote comparison of response of matured PLBs to different PGR combination while uppercase letters represent response of PLBs matured on different sucrose concentrations to one PGR

MULTIPLICATION

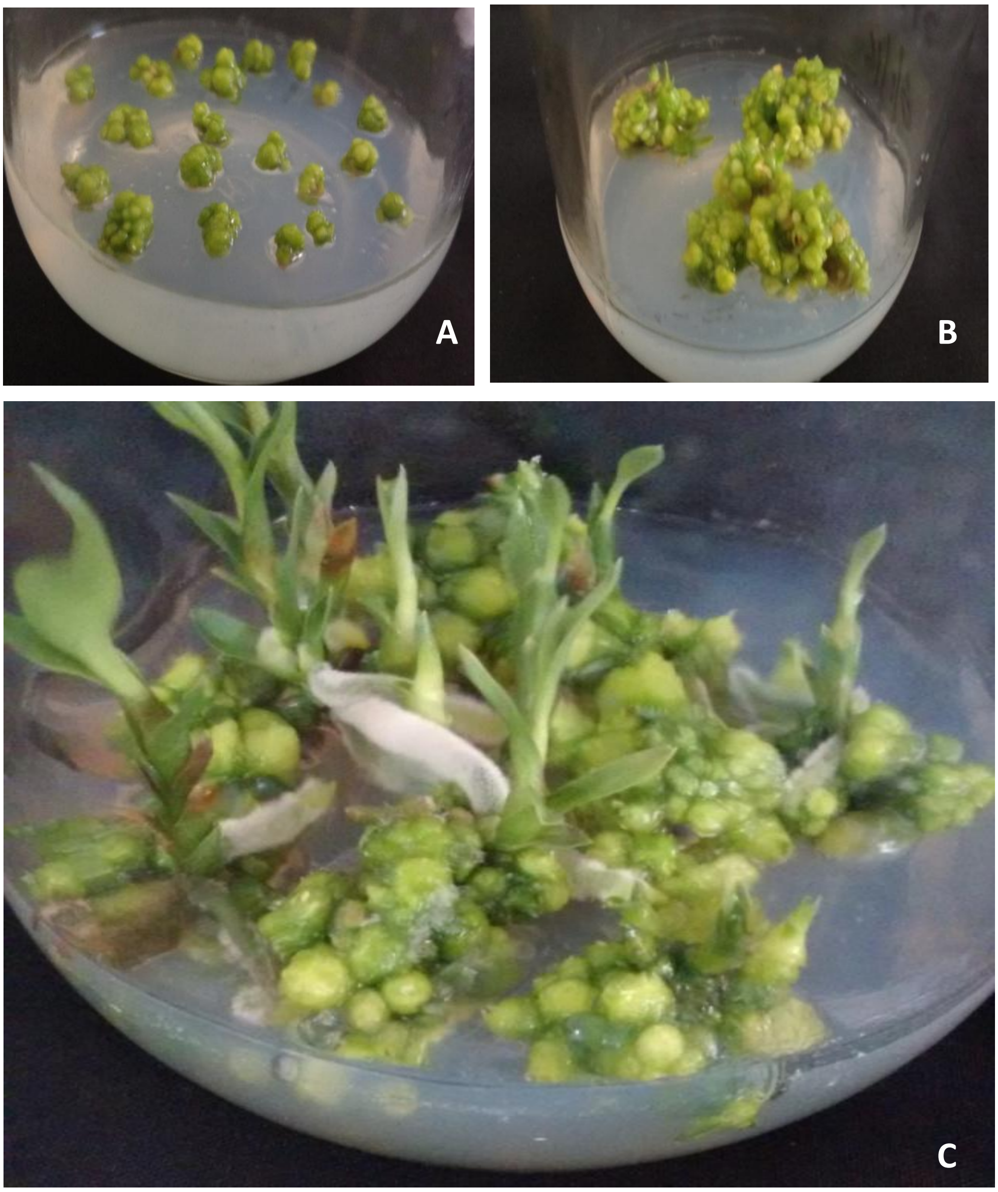


Fig 6 **A-C**: Mutiplication of *Cymbidium* cultures using PLBs **A**-single PLBs **B**- PLBs in clusters after 21 days **C**- 90 days of culture in MS basal medium supplemented with 2.5µM BAP

MATURATION AND GERMINATION

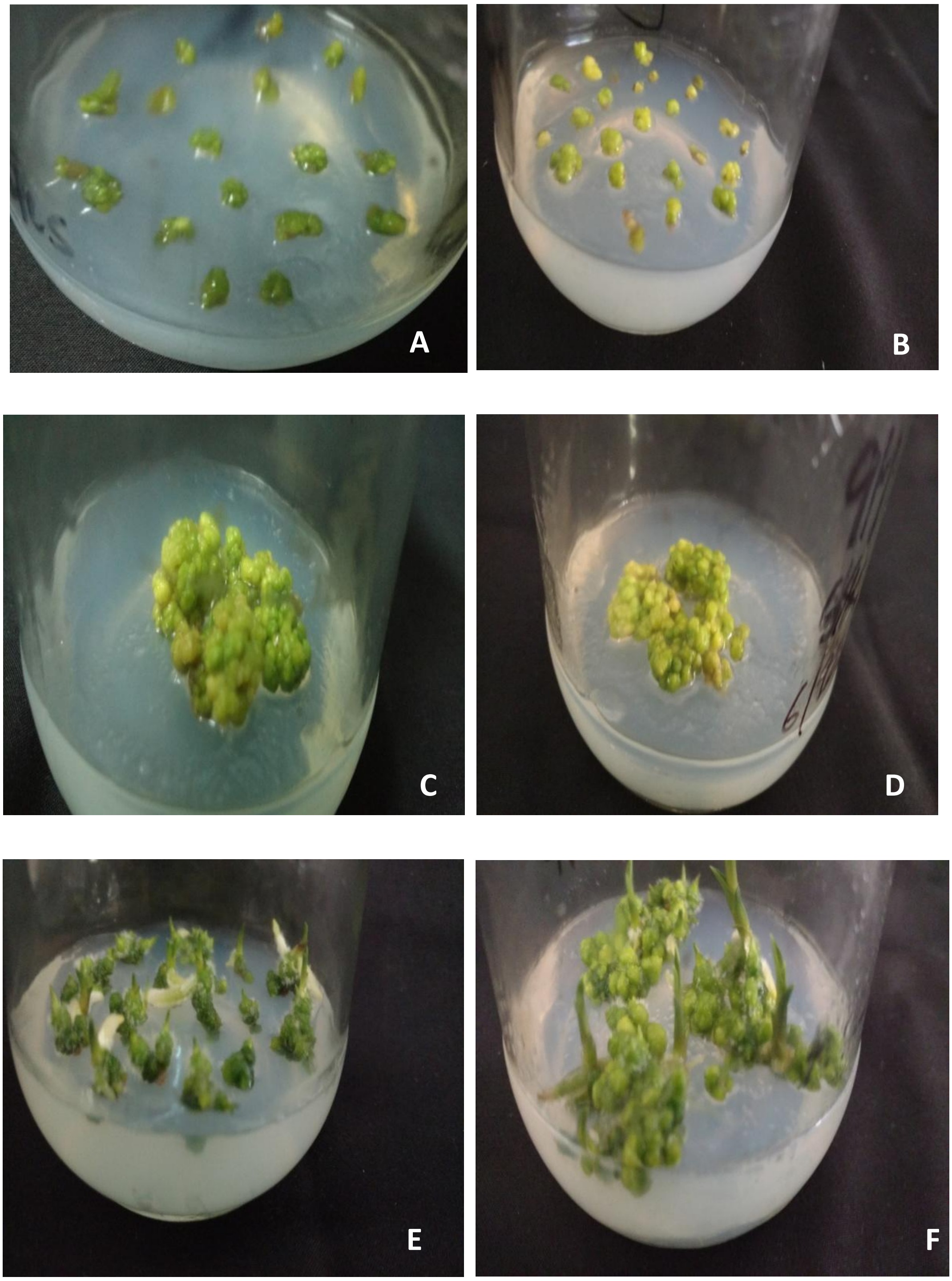


Fig. 7: A-D Maturation of PLBs on high energy sucrose supplemented MS basal media A- unmatured single PLBs B- matured single PLBs C- unmatured PLBs in cluster D- matured PLBs in cluster

E-F Germination of PLBs on conversion medium E- single PLB germination F- germination in cluster

Table 13: The effect of different concentrations of GA_3 and BAP on conversion of PLBs matured on 261 mM sucrose

Basal MS with different PGR Concentrations (µM)		Percent PLBs converting into shoots (Mean± S.E)
GA_3	BAP	
-	-	04.773^{bc} ± 02.762
1	-	70.448^{a} ± 05.719
2.5	-	60.308^{a} ± 12.955
5	-	65.450^{a} ± 10.523
-	1	02.075^{c} ± 02.075
1	1	04.545^{bc} ± 04.545
2.5	1	06.060^{bc} ± 04.285
5	1	25.458^{b} ± 06.561
-	2.5	00.000^{c} ± 00.000
1	2.5	02.380^{c} ± 01.683
2.5	2.5	54.315^{a} ± 11.023
5	2.5	51.490^{a} ± 05.441

In the gradient experiment, PLBs were inoculated into basal MS medium supplemented with different combinations of PGRs (GA_3 and BAP) and results were recorded after 60 days of culture. It was found that the highest (70.44%) PLBs converted into shoots were obtained in the basal MS medium supplemented with 1 µM GA_3 followed by 5 µM and 2.5 µM GA_3 and the lowest (2.07%) PLBs converted into shoots were obtained in basal MS medium supplemented with 1 µM BAP and no shoots were obtained in basal MS medium supplemented with 2.5 µM BAP.

SYNTHETIC SEEDS

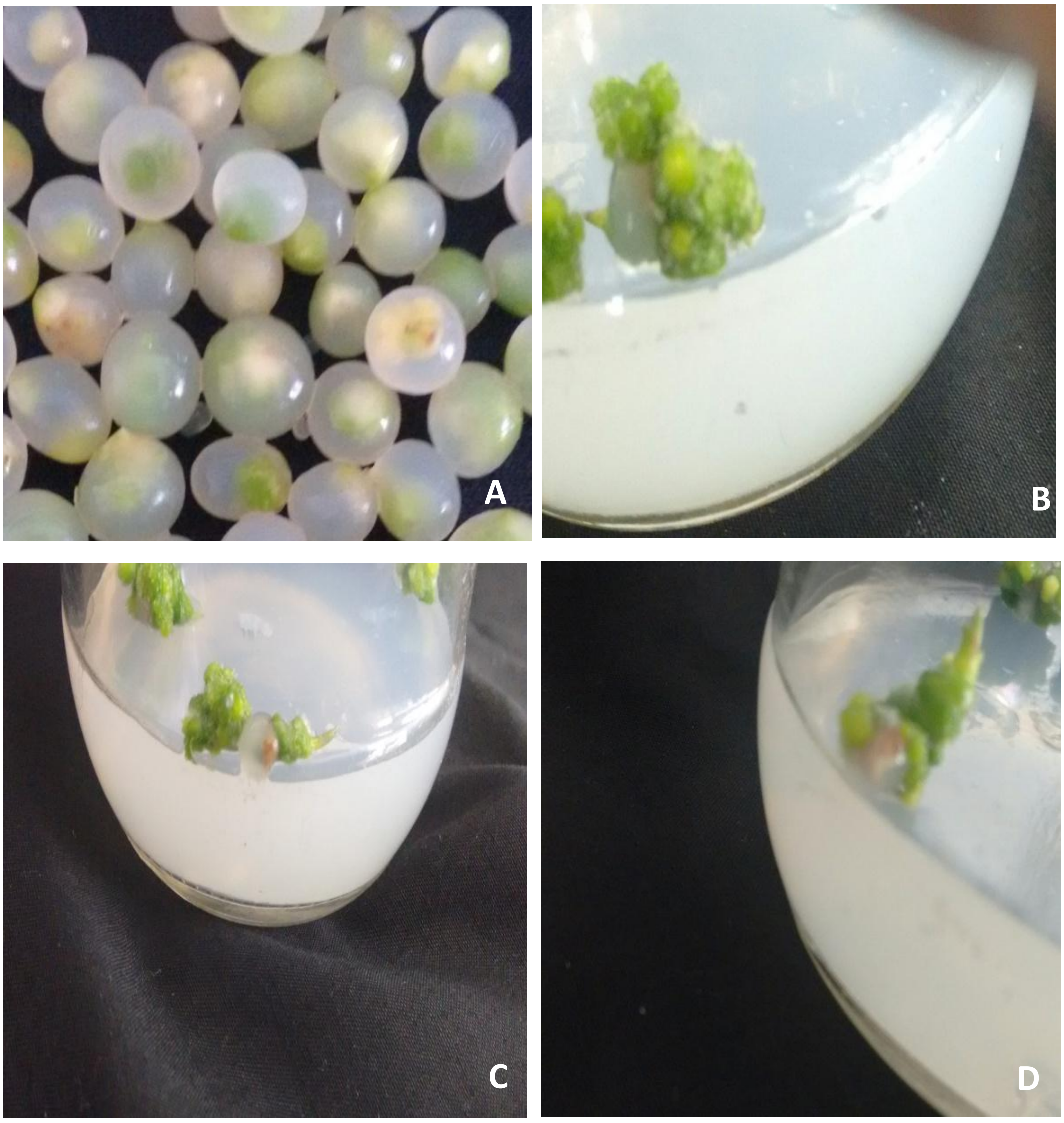


Fig 8 A-D Sodium alginate based synthetic seeds of *Cymbidium giganteum*× *C.elegans*

A- Synthetic Seeds B- PLB encapsulated seed multiplied C-D Germinated Seed

Table 14: Effect of storage period on germination and multiplication of encapsulated PLBs stored at different temperatures

Storage period (days)	**Percent number of seeds germinated after 65 days of culture**		**Percent number of seeds multiplied after 80 days of culture**		**Percent number of viable seeds after 80 days**	
	4ºC	25ºC	4ºC	25ºC	4ºC	25ºC
10	3.33± 11.25	0.00± 0.00	96.66 ± 3.33	60± 11.54	93± 4.21	36± 8.02
20	0.00± 0.00	0.00± 0.00	60 ± 5.16	46.66±15	50± 4.47	30± 10
30	0.00± 0.00	0.00± 0.00	36.66 ± 3.33	40 ± 0.00	50± 6.83	53.3± 8.43
40	6.66± 4.21	0.00± 0.00	36.66± 8.02	03± 03	46.66± 8.43	20± 7.30
Control (0 day)	30± 11.25		96.66± 3.33		96.66± 3.33	

Data represents Means ± S.E (n=6) with 5 seeds in each culture vessel

The effect of temperature on the synthetic seeds was studied at different durations of days (10, 20, 30, 40) at 4ºC and 25ºC. No germination of seeds stored at 25ºC was observed after 10, 20, 30 and 40 days of culture and very little germination 3.33% and 6.66% was observed in seeds stored at 4ºC after 10 and 40 days respectively. However, the multiplication of encapsulated PLBs from these seeds was observed when cultured on the conversion medium and highest (96.66) percent number of PLBs multiplied following storage of seeds at 4ºC after 10 days of culture. Similar multiplication was observed in the seeds stored after 30 and 40 days of culture. The highest number (93%) of viable seeds was observed in seeds stored at 4ºC for 10 days. The viability of seeds decreases after every 10 days of interval upto 40 days at 4ºC. No such sequential increase or decrease was observed at 25ºC.

CHAPTER 5

DISCUSSION

The present study was focussed on multiplication of PLBs of newly developed F1 interspecific hybrid of *Cymbidium giganteum* × *C.elegans* and to study the effect of sucrose and mannitol concentrates on the maturation of PLBs of *Cymbidium* hybrid. Following the maturation, conversion experiment was conducted on the matured PLBs using different PGRs. Sodium alginate based synthetic seeds were then prepared using PLBs as explants and these were then stored for different durations (0, 10, 20, 30 and 40 days) at two different temperatures i.e. 25ºC and 4ºC. Later, the effect of two different storage temperatures (0ºC and 25ºC) on the conversion of stored synthetic seeds were studied following storage for different periods and conversion efficiency was checked.

Initially the PLBs of *in vitro* grown *Cymbidium* hybrid were multiplied on MS basal medium supplemented with 2.5 μM BAP. Importance of BAP supplemented MS medium was reported on shoot multiplication in nodal explants of orchid *Vanilla planifolia* (Kalimuthu et al. 2006).

The maturation experiment was conducted using PLBs as single and in clusters. These PLBs were inoculated on MS basal medium supplemented with various concentrations of sucrose (87 mM, 174 mM, 261mM and 348 mM) or mannitol (0 mM, 82.5 mM, 165mM and 247 mM). The maturation was observed after 30 and 40 days of culture, both in sucrose and mannitol supplemented MS basal medium. The best maturation of PLBs was observed, when inoculated as single piece on basal MS (40.96%) containing 261 mM sucrose after 30 and 40 days of culture (Table 1). At 87 mM sucrose, the PLBs remained green in colour and at 348 mM sucrose concentration, PLBs turned brown resulting in the death. But at 261 mM sucrose concentration green PLBs turned into yellow colour showing their maturation. Similarly, when PLBs were inoculated in clusters in sucrose or mannitol supplemented medium, highest maturation was observed at 261 mM sucrose concentration and 247 mM mannitol concentration (Table 3, 4). But the PLBs matured on sucrose turned dark yellow in comparison to PLBs matured on mannitol supplemented basal medium. Maturation in sucrose was better than mannitol. Similar results were found by Tremblay and Tremblay (1995) in *Picea mariana* in which it was observed that the medium containing higher concentration of sucrose (7-12%) was better for maturation of somatic embryos than the mannitol. Also, Trigiano and Gray (2010) reported that high sucrose was successful for maturation.

After maturation, the conversion experiment was carried out using matured PLBs (at all concentrations of sucrose or mannitol). These 30 and 40 day matured PLBs (as single piece and in clusters) were then inoculated separately on four different PGRs [0.1 μM BAP, 1 μM GA_3, MS and (0.1 μM BAP + 1 μM GA_3)] supplemented MS basal medium. Results were recorded after 60 days of culture. Best (86.93%) of germination was observed in 40 day matured PLBs (as single piece) on 87 mM sucrose which were then cultured on 1 μM GA_3 (Table 9) and 81.2% germination in 30 day matured PLBs (as single piece) on 87 mM sucrose which were then cultured on 1 μM GA_3 (Table 5). Also, 74% germination in 30 day matured PLBs (in clusters) on 87 mM sucrose which were then cultured on 1 μM GA_3 and 66% germination in 40 day matured PLBs (in clusters) on 87 mM sucrose which were then cultured on 0.1 μM BAP + 1 μM GA_3 combination. 78.12% germination was observed in 30 day matured PLBs (as single piece) which were cultured on 0.1 μM BAP + 1 μM GA_3 combination with no mannitol (Table 7) and 82.77% germination was observed in 30 day matured PLBs (in clusters) on 1 μM GA_3 supplemented MS medium with no mannitol (Table 8). This shows that mannitol is not required for maturation as compared to sucrose in germination experiments.

The effect of 12 different combinations of GA_3 and BAP on conversion of matured PLBs was studied. The PLBs were matured on 261 mM sucrose supplemented medium and were then cultured on the different combinations of GA_3 and BAP in which the highest (70.448%) of shoot germination was observed on basal MS supplemented with 1 μM GA_3 followed by MS supplemented with 5 μM GA_3 (65.450%), while the lowest (2.07%) of shoot germination was observed on basal MS medium supplemented with 1 μM BAP and germination was not recorded on basal MS supplemented with 2.5 μM BAP.

Importance of GA_3 supplemented MS basal medium for shoot germination has been reported in *Cajanus cajan* (Srivastava and Raghav 2014).

After maturation and germination, synthetic seeds of *Cymbidium giganteum* × *C. elegans* were prepared using 261 mM sucrose matured PLBs and 3% sodium alginate as gelling agent and 125 mM Calcium chloride as complexing agent was used for the encapsulation purpose (Ranjan 2015). Synthetic seed production have been reported in Orchids like *Dendrobium densiflorum*, *Avanda* Wan Chark Kuan 'Blue' × *Vanda coeruleae* ex. Lindl. (AV) Gantait et al. (2012). Mohanty and Das (2013) prepared synthetic seeds of *Dendrobium densiflorum* using different concentrations of sodium alginate 2, 3, 4 and 5% (w/v) and calcium chloride in the range of 50, 100 or 150 mM (w/v).

Storage experiment was conducted on the encapsulated seeds of *Cymbidium giganteum*× *C.elegans.* The seeds were stored in tissue culture bottles and were kept at two different temperatures i.e. 4ºC (cold temperature) and at 25ºC (room temperature). After that the seeds were cultured on the conversion medium (MS basal supplemented with 1 μM GA_3) after different durations of 10, 20, 30 and 40 days. The effect of two different temperatures (4ºC and 25ºC) on conversion of PLBs was studied. Higher conversion frequency was observed in PLBs stored at 4ºC. It was also observed that the seeds stored at 4ºC were viable for longer periods of time in comparison to seeds stored at 25ºC.

Similar storage experiments were performed on different orchids like *Dendrobium* 'Shavin White', *Dendrobium densiflorum*. Bustam et al. (2013) studied the storage of encapsulated PLBs and kept the seeds at various temperatures of 4ºC, 10ºC , 25ºC and 30ºC for different durations (15, 45, 75, 105 and 135 days) and after that these PLBs were cultured on to ½ MS basal medium for 8 days for conversion.

Different parameters like germination and conversion were studied in *Dendrobium* 'Shavin White' (Bustam et al. 2013) after the storage experiment and it was found that PLBs (with shoot) were germinated earlier than PLBs without shoot and it took very less time for germination (12-13 days).

CHAPTER 6

CONCLUSION

Cymbidium orchid has been found to be one of the important ornamental plants. From the present research work it was found that the energy sources and PGRs play an important role in maturation, germination of PLBs respectively. It was also observed that storage temperature and duration influence synthetic seed germination in *Cymbidium*.

In the present study,

1. Multiplication was successfully carried out from the in vitro grown cultures of *Cymbidium* hybrid on MS medium supplemented with 2.5µM BAP
2. Best maturation was observed on MS basal medium supplemented with 261 mM sucrose
3. Maximum conversion of PLBs matured on 87 mM sucrose was achieved on medium supplemented with 1 µM GA_3
4. Successful encapsulation of PLBs matured on 261 mM sucrose was done in 3% alginate gel.
5. Storage experiment was successfully conducted and it was observed that the seeds stored at 4ºC were viable for longer period as compared to seeds stored at 25ºC
6. Conversion efficiency of stored synthetic seeds was checked with higher conversion frequency in the encapsulated PLBs stored at 4ºC
7. Mutiplication of encapsulated PLBs was also observed in the stored seeds, when cultured on the conversion medium with higher multiplication rate in seeds stored at 4ºC

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ANNEXURE 1

Composition of MS medium as described by Murashige and skoog, 1962

Composition	Amounts (mg/l)
1. Macronutrients (Hi media)	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_20$	440
$MgSO_4.7H_20$	370
KH_2PO_4	170
2. Micronutrients	
$MnSO_4.4H_2O$	16.90
$Fe_2EDTA. 2H_2O$	27.80
$ZnSO_4. 7H_2O$	08.60
H_3BO_4	06.20
KI	00.83
$Na_2MoO_4.2H_2O$	00.25
$CoCl_2.6H_2O$	00.25
$CuSO_4.5H_20$	0.025
3. Vitamins	
Myoinositol	100
Glysine	2.0
Nicotinic Acid	0.5
Pyridoxine HCL	0.5
Thiamine HCL	0.1
4. Sugar	3%
5. Agar	8000