CHAPTER 5

PRODUCTION OF SYNTHETIC SEEDS OF *Gerbera jamesonii* Bolus ex. Hook f

5.1 EXPERIMENTAL AIMS

The development of synthetic seed technology brings up a new prospect in agriculture industry. Production of synthetic seeds is an alternative method for mass propagation that enables multiplication of seedless plants, transgenic plants, plants with problems in seed propagation and many others. The main objective of micropropagation technique is to ensure continuous supply of desired plant species. In some plants and crop species, propagation through germination of seeds has not been successful. This is mainly due to the seed size, heterozygosity of the seeds, presence of reduced endosperm of the seed and the requirement of mycorrhizal fungi association for germination (eg. Orchids) and also in some seedless varieties in crop plants like grapes, watermelon, etc.

Reproduction by asexual means of *Gerbera* is known to be time consuming and very expensive. Development of artificial seed production is effective and acts as an important alternative method of propagation in several commercially important plant species with high commercial values. Synthetic seed production has many advantages over conventional propagation. Plants could be produced in large scale with high volumes. Consequently genetic uniformity and stability of the plant could be maintained. Due to the sterility of the synthetic seeds, it could be easily transported from one country to another and has potential for long term storage without losing its viability.

The production cost for each plantlet is lower compared to normal propagation method. Each synthetic seed could produce rapid multiplication of new plants in a short period of time. Synthetic seeds technology also serves as a channel for new plant lines produced through biotechnological methods to be delivered directly to the greenhouse or field. Plant propagation through germination of seeds obtained from mother plant of *G. jamesonii* is very limited and this method is not a favorable way of propagation for commercialization. *G. jamesonii* is known to be a temperate grown plant. Seeds of *Gerbera* are expensive and needs to be imported and this of course requires high transportation cost.

Thus, production of synthetic seeds of *G. jamesonii* is a timely effort in promoting this plant species as a commercially important ornamental in Malaysia. Synthetic seeds could be produced through the encapsulation of micro shoots and somatic embryos. Synthetic seeds of many plant species have been produced mainly from the encapsulation of somatic embryos. However, synthetic seeds could also be produced through the encapsulation of micro shoots.

Objective of this study was to investigate and establish the efficient system for the production of synthetic seed of *G. jamesonii in vitro*. Synthetic seeds production ensures an alternative to mass propagation of this ornamental plant species. Micro shoots were used as propagules in this experiment. Ideal formation of *Gerbera* synthetic seeds in the aspect of size, shape and solidity of the seeds were determined. The most suitable concentration of sodium alginate ($NaC_6H_7O_6$) and calcium chloride dehydrate

($CaCl_2.2H_20$) solution determines the optimum encapsulation matrix. Germination rate of *Gerbera* synthetic seeds were identified and thus the most suitable germination medium would be identified. To this date, there is still no other documentation and reports on the production of synthetic seed of *G. jamesonii*.

5.2 MATERIALS AND METHODS

5.2.1 Production of Micro Shoots

Micro shoots were identified as the best propagule for this experiment (Figure 5.1). From the previous experiments, micro shoots were obtained when petiole explants were cultured on MS medium supplemented with 1.0-3.0 mg/l BAP after 6 weeks of culture. Micro shoots sized ranging from 3.0-5.0 mm were excised from the explants and ready to be encapsulated to form artificial seed.

5.2.2 Preparation of MS Basal Medium

MS basal medium was prepared using MS stock solution (refer to 2.2.5) at pH 5.8. Artificial seeds formed were rinsed using this solution. MS basal medium prepared were autoclaved for 21 minutes at 121 ^{o}C prior to obtaining sterile solution.

5.2.3 Preparation of Sodium Alginate Solution ($NaC_6H_7O_6$)

In this experiment, sodium alginate that consists of alginic acid, which is a type of sodium salt, was used as the encapsulation matrix. Sodium alginate solutions at 1.0%, 2.0%, 3.0%, 4.0% and 5.0% concentrations were prepared. This solution was prepared using a heating method by Fabre and Dereuddre (1990). Sodium alginate solution was prepared in 100ml MS basal medium without the additions of calcium chloride dehydrate ($CaCl_2.2H_20$). To prepare 1.0% sodium alginate solution, 1.0 g of sodium alginate powder was added slowly in MS basal medium without calcium chloride dehydrate

($CaCl_2.2H_20$) to allow it to dissolve completely and followed by the addition of 3.0 g sucrose, autoclaved for 21 minutes at 121 ^oC. Sterile sodium alginate solution was used as an encapsulation matrix for the production of artificial seeds.

5.2.4 Preparation of Calcium Chloride Dehydrate Solution

Calcium chloride dehydrate ($CaCl_2.2H_2O$) acts as complexing solution that allows the hardening of encapsulated matrix. Different concentrations of CaCl2.2H2O (25-125 mM) were prepared in liquid MS medium. To prepare 25mM $CaCl_2.2H_2O$ solution, 0.9189 g of $CaCl_2.2H_2O$ powder was added into 250 ml MS liquid basal medium. This solution was autoclaved for 21 minutes at 121 ^oC.

5.2.5 Encapsulation Techniques and Formation of Synthetic Seeds

Synthetic seeds were formed using encapsulation technique. This technique involved the usage of sodium alginate ($NaC_6H_7O_6$) and calcium chloride dehydrate solution ($CaCl_2.2H_2O$). This technique was introduced by Lynch (2002). In this technique, micropipette was used as an important tool to form the beads. The diameter of pipette tip was 5.0 mm. Micro shoots were first mixed with sodium alginate solution. Encapsulation was accomplished when each micro shoot was drawn up with some of the encapsulation matrix (sodium alginate solution) and then dropped into the $CaCl_2.2H_2O$ solution. This process was done repeatedly. Each drop has to contain only one micro shoot (explant). Round shaped beads containing micro shoots were formed when the droplets were dropped in the $CaCl_2.2H_2O$ solution. $CaCl_2.2H_2O$ solution needs to be

agitated slowly to avoid all droplets formed fused with each other. During this process, ion exchange reaction between sodium ion (Na^+) and Calcium ion (Ca^+) occurred. This reaction is important in developing insoluble sodium alginate. The resulting beads containing explants were left agitated in the $CaCl_2.2H_2O$ solution for 30 minutes. The beads were sieved using a nylon mesh. All encapsulated beads were rinsed three times with sterile liquid MS medium.

5.2.5.1 Sodium Alginate Solution ($NaC_6H_7O_6$) and Calcium Chloride Dehydrate Solution in Different Concentrations

Five different concentrations of sodium alginate solution at 1.0%, 2.0%, 3.0%, 4.0% and 5.0% were prepared respectively (section 5.2.3). Five different concentrations of $CaCl_2.2H_2O$ were also prepared at 25 mM, 50 mM, 75 mM, 100 mM and 125 mM. The encapsulation of beads was tested using all five concentrations of sodium alginate solution. The beads formed were soaked in $CaCl_2.2H_2O$ at different concentrations for 30 minutes. Results were obtained based on the observations on the shape of beads formed. Thirty replicates were used in each treatment. From this experiment the optimum sodium alginate and calcium chloride dehydrate solution to form the ideal beads were identified. Synthetic seeds formed were germinated on MS solid and liquid media.

5.2.5.2 Preparation of Encapsulation Matrix in Different Solution

Encapsulation matrix was prepared by dissolving sodium alginate powder in MS basal liquid medium (without $CaCl_2.2H_2O$) with the addition of sucrose. In this experiment, sodium alginate powder was dissolved in 4 different solutions which are;

1. MS basal liquid medium
2. MS basal liquid medium (without $CaCl_2.2H_2O$)
3. MS basal liquid medium (without $CaCl_2.2H_2O$) added with 2.0 mg/l BAP + 0.5 mg/l NAA (optimum hormone for regeneration of shoots)
4. Distilled water

Four different types of encapsulation matrix were prepared using these solutions. Three percent of sodium alginate and 3.0 g sucrose were dissolved in each of the solution. Micro shoots were then encapsulated using the encapsulation technique. Beads were cultured and germinated on MS basal solid medium in the culture room at 25 ± 1 ^{o}C and 16 hours light and 8 hours dark. Observations were made daily and all results were recorded. Thirty replicates were used in each treatment.

5.2.6 Germination of Synthetic Seeds on Various Sowing Media

The success of the production of synthetic seeds of *G. jamesonii* were determined by absorbing and recording the ability of the seeds to germinate, grow and finally acclimatized and developed into normal plants. Synthetic seeds formed from the

encapsulation of micro shoots composed of Ca-free MS with the addition of 3.0 % sodium alginate, 3.0 % sucrose and supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA were germinated on 3 different types of sowing media. MS basal liquid medium, sterile garden soil and sterile vermiculite were used in this experiment. The most suitable sowing medium was identified.

5.2.7 Low Temperature Storage

A set of encapsulated explants (artificial seeds) were preserved at low temperature (4 ± 1 ^{o}C) for 15, 30, 60, 90,120, 150 and 180 days under *in vitro* condition. After the storage period, the beads were cultured on MS basal solid medium. The survival rates for germination of preserved beads were recorded.

5.2.8 Data Analysis

Data obtained were analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at p=0.05

5.3 RESULTS

5.3.1 The Effect of Different Concentrations of Sodium Alginate and Calcium Chloride Dehydrate Solution on Bead Formation

Different concentrations of sodium alginate and calcium chloride dehydrate solution were used to identify the optimum concentration of these solutions to form ideal beads. From this experiment, it was observed that sodium alginate at 3.0% and 4.0% formed optimal, firm, uniform and round shaped beads when the beads were left to be hardened in 100 mM (Figure 5.2) and 125 mM calcium chloride dehydrate solution for 30 minutes (Table 5.1). Beads formed using 2.0 % sodium alginate was fragile with no definite shape while 5.0% and 6.0% sodium alginate formed rigid, stiff and unbreakable beads. Thus these concentrations were not suitable for the production of beads to form synthetic seeds.

5.3.2 Germination of *Gerbera* Synthetic Seeds on Different Germinating Media

In this experiment, micro shoots (Figure 5.1) were encapsulated using 3.0% and 4.0% sodium alginate (Figure 5.3). Meanwhile 100 mM and 125 mM of calcium chloride dehydrate were used as complexing solution to harden the encapsulated matrix. Synthetic seeds produced were germinated in two sowing media, MS solid and liquid media. Seeds produced using 3.0 % sodium alginate and soaked in 100 mM and 125 mM $CaCl_2$.H2O solution germinated both on MS solid and liquid media after 5 days.

Percentage of germination rates of beads soaked in 100 mM was 72.1 ± 0.8% on MS solid media and 69.5 ± 1.2% on MS liquid media whereas the survival rates of the synthetic seeds were 73.6 ± 1.0% on MS solid media and 70.0 ± 1.0 % on MS liquid media (Table 5.2). Synthetic seeds immersed in 125 mM CaCl2.H2O solution yielded germination rate of 70.5 ± 1.3 % and 67.0 ± 0.5% when germinated on MS solid and liquid media, respectively.

Beads formed using 4.0% sodium alginate and soaked in 100 mM $CaCl_2.H_2O$ germinated after 7 days both on MS solid and liquid media. The germination rates were 64.3 ± 0.9 % when germinated on MS solid media and 58.3 ± 1.0% on MS liquid media. Beads immersed in 125 mM germinated at 46.6 ± 1.1 % and 43.0 ± 0.7% on MS solid and liquid media. Beads formed with 4% sodium alginate and soaked in 100 mM $CaCl_2.H_2O$ showed 69.2 ± 1.5% survival after 30 days of germination on MS solid media and 57.2 ± 1.3.0% on MS liquid media whilst in 125 mM showed 62.5 ± 1.2% and 54.6 ± 1.2% survival in MS solid and liquid media respectively.

Table 5.1: Effect of different concentrations of sodium alginate ($NaC_6H_70_6$) and calcium chloride ($CaCl_2.H_2O$) on bead formation. Thirty replicates were prepared for each treatment.

$CaCl_2.2H_2O$ (mM)	Sodium alginate concentration (%)				
	2.0	3.0	4.0	5.0	6.0
25	+	+ + +	+ + +	+ + +	+ + +
50	+	+ + +	+ + +	+ + +	+ + +
75	+ +	+ + +	+ + +	* * *	* * *
100	+ +	+ + + +	+ + + +	* * *	* * *
125	+ +	+ + + +	+ + + +	* * *	* * *

\+ Very fragile bead with no definite shape
+ + Fragile beads with no definite shape
+ + + Soft, solid and uniform shape
+ + + + Optimal, firm, uniform and round shape
* * * Rigid, stiff and unbreakable beads

Table 5.2: Effect of different concentration of sodium alginate ($NaC_6H_70_6$) and calcium chloride ($CaCl_2.H_2O$) solution on germination of *Gerbera jamesonii* synthetic seed on solid and liquid media. Thirty replicates were used in each treatment.

Sodium Alginate and Calcium Chloride Solution ($CaCl_2.H_2O$)	Period of Germination (Day)		Germination Rate (%) (10 days)		Survival Rate (%) (30 days)	
	MS Solid Media	MS Liquid Media	MS Solid Media	MS Liquid Media	MS Solid Media	MS Liquid Media
3.0% Sodium Alginate and 100 mM $CaCl_2.H_2O$	5	5	$72.1 \pm 0.8_a$	$69.5 \pm 1.2_b$	$73.6 \pm 1.0_a$	$70.0 \pm 1.0_b$
3.0% Sodium Alginate and 125 mM $CaCl_2.H_2O$	5	5	$70.5 \pm 1._{3a,b}$	$67.0 \pm 0.5_{b,c}$	$70.3 \pm 0.8_b$	$64.5 \pm 0.5_{b,c}$
4% Sodium Alginate and 100 mM $CaCl_2.H_2O$	7	7	$64.3 \pm 0.9_{b,c}$	$58.3 \pm 1.0_c$	$69.2 \pm 1.5_{b,c}$	$57.2 \pm 1.3_{c,d}$
4% Sodium Alginate and 125 mM $CaCl_2.H_2O$	8	8	$46.6 \pm 1.1_{c,d}$	$43.0 \pm 0.7_d$	$62.5 \pm 1.2_c$	$54.6 \pm 1.2_d$

Mean ± SE, n=30. Mean with different letters differ significantly at p=0.05

Figure 5.1: Micro shoots of *Gerbera jamesonii* cultured on MS medium supplemented with 3.0 mg/l BAP

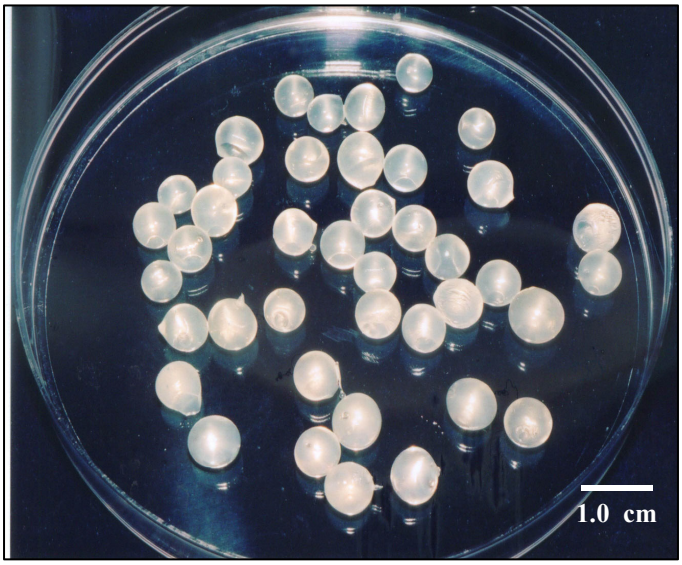

Figure 5.2: Ideal beads formed when encapsulation matrix was composed of Ca-free MS basal medium added with 3.0% sodium alginate and 3.0% sucrose and soaked in 100 mM $CaCl_2.H_2O$.

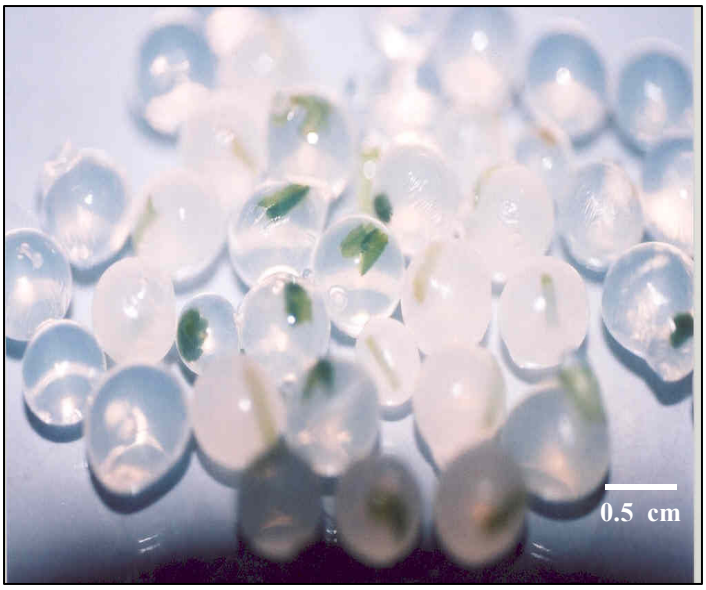

Figure 5.3**:** Encapsulated micro shoots of *Gerbera jamesonii*

5.3.3 Encapsulation Matrix in Different Solution

For the production of synthetic seeds, the encapsulation matrix was made when sodium alginate powder was dissolved in Ca-free MS basal liquid medium with the addition of sucrose that acts as carbon source. Encapsulation matrix was formulated when sodium alginate powder was dissolved in 4 different solutions. Germination and survival rates of *Gerbera* synthetic seeds were observed. Encapsulation matrix composed of Ca-free MS with the addition of 2.0 mg/l BAP and 0.5 mg/l NAA resulted in 74.5 ± 2.6% of germination rate and 75.0 ± 0.5% survival rate (Table 5.3). Encapsulation matrix composed of Ca-free MS without the addition of hormones yielded 72.1 ± 0.8% germination rate. The survival rate after 30 days of germination was 73.6 ± 1.2%. Encapsulation matrix composed of distilled water only produced 20.2 ± 0.8% of germination rate and survived with a percentage of 16.3 ± 2.1.

5.3.4 Effect of Different Types of Sowing Media on Germination of Synthetic Seed

In general, all 3 types of sowing media were able to be used as germinating media. 100 ± 0.0% of germination was obtained when synthetic seeds of *Gerbera* were germinated on MS basal solid medium (Figure 5.4). Sterile garden soil and vermiculite both germinated at 90.6 ± 0.7% and 70.3 ± 1.3%, respectively (Table 5.4).

Table 5.3: Growth response of micro shoots of *Gerbera jamesonii* encapsulated in different encapsulation matrix.

Encapsulation matrix	Percentage of Germination rate of Encapsulated micro shoots (10 days)	Percentage of Survival rate of Encapsulated micro shoots (8 weeks)
MS	$70.5 \pm 1.2_{b}$	$69.5 \pm 0.7_{b}$
Ca-free MS	$72.1 \pm 0.8_{b}$	$73.6 \pm 1.2_{b}$
Ca-free MS + 2.0 mg/l BAP + 0.5 mg/l NAA	$74.5 \pm 2.6_{b,c}$	$75.0 \pm 0.5_{b,c}$
Distilled water	$20.2 \pm 0.8_{a}$	$16.3 \pm 2.1_{a}$

Mean ± SE, n=30. Mean with different letters in the same column differ significantly at p=0.05

Table 5.4: Effect of different sowing media on germination rate of synthetic seeds of *Gerbera jamesonii*.

Sowing media	No. of germinated seeds	Germination rate (%)
	Encapsulated micro shoot	Encapsulated micro shoot
MS basal	$30.0 \pm 0.0_{a}$	$100 \pm 0.0_{a}$
Garden soil	$27.2 \pm 1.2_{a,b}$	$90.6 \pm 0.7_{a,b}$
Vermiculite	$21.6 \pm 0.4_{b,c}$	$70.3 \pm 1.3_{b,c}$

Mean ± SE, n=30. Mean with different letters in the same column differ significantly at p=0.05

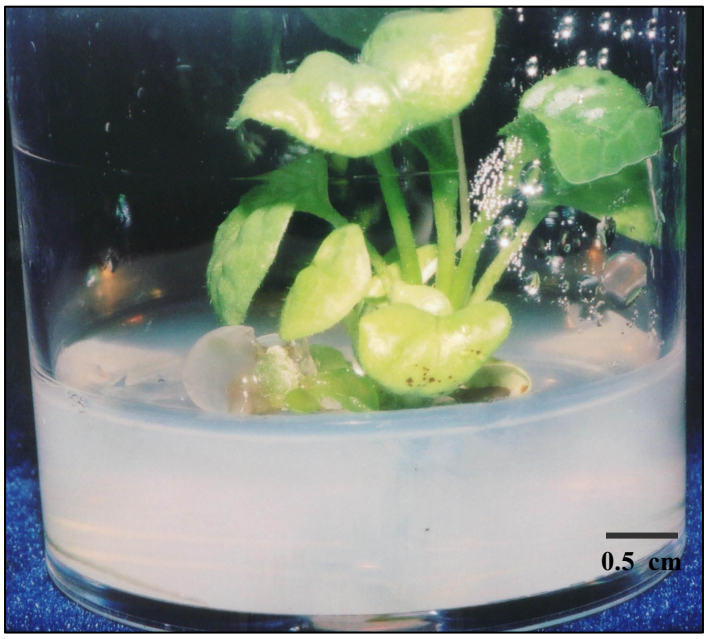

Figure 5.4: Five-week-old Plantlet obtained from germination of synthetic seed of *Gerbera jamesonii*

5.3.5 Low Temperature Storage

Synthetic seeds of *G. jamesonii* preserved at 4 ± 1 ^{o}C with different storage periods were germinated on MS basal solid medium. It was observed that the longer the seeds being stored, the germination rate was reduced. Synthetic seeds incubated for 30 days germinated at 91.7 ± 0.6% (Table 5.5). Meanwhile, seeds stored for 60, 90 and 120 days produced 91.0 ± 0.8%, 77.0 ± 1.0% and 53.3 ± 1.2% of germination. The lowest germination rate (14.3 ± 0.7%) was observed when the seeds were preserved for 180 days. Generally synthetic seeds of *Gerbera* were able to germinate when preserved at low temperature with certain storage period but exhibited lower germination rates as the storage period increased.

Table 5.5: Effect of storage period (days) at 4 ± 1 ^{o}C on germination of synthetic seeds of *Gerbera* on germination MS basal medium.

Period of storage (days)	No. of germinated seeds	Germination rate (%)
	Encapsulated micro shoot	Encapsulated micro shoot
0	$28.0 \pm 1.2_{a}$	$93.3 \pm 0.5_{a}$
30	$27.5 \pm 1.1_{a,b}$	$91.7 \pm 0.6_{a,b}$
60	$27.3 \pm 0.8_{a,b}$	$91.0 \pm 0.8_{a,b}$
90	$23.1 \pm 1.3_{b,c}$	$77.0 \pm 1.0_{b,c}$
120	$16.0 \pm 0.9_{c}$	$53.3 \pm 1.2_{c}$
150	$7.0 \pm 1.5_{d}$	$23.3 \pm 0.8_{d}$
180	$4.3 \pm 0.7_{d,e}$	$14.3 \pm 0.7_{d,e}$

Mean ± SE, n=30. Mean with different letters in the same column differ significantly at p=0.05

5.4 SUMMARY OF RESULTS

1. Sodium alginate ($NaC_6H_7O_6$) at 3.0% with 100 mM calcium chloride dehydtrate ($CaCl_2.2H_2O$) solutions were the optimum concentrations to form encapsulation matrix for the production of synthetic seeds of *G. jamesonii*. The beads formed were firm, easy to germinate, uniform and round shaped.

2. Germination rate of 72.1 ± 0.8% was obtained when synthetic seeds of *Gerbera* were germinated on MS solid media while 69.5 ± 1.2% on MS liquid media. Synthetic seeds of *Gerbera* germinated after 5 days on MS solid and liquid media.

3. Encapsulation matrix for production of synthetic seeds were optimized when 3.0% sodium alginate was added with Ca-free MS with the addition of 3.0% sucrose supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA which was the optimum hormones for shoot regeneration. The germination and survival rates after 10 days of encapsulation process were 74.5 ± 2.6% and 75.0 ± 0.5%, respectively.

4. MS basal solid medium, sterile garden soil and vermiculite were found to be suitable substrates for germinating *Gerbera* synthetic seeds. Germinating rate of *Gerbera* synthetic seed was 100.00 ± 0.0% on MS basal solid medium, followed by 90.6 ± 0.7% on sterile garden soil and 70.3 ± 1.3 on sterile vermiculite.

5. Synthetic seeds of *G. jamesonii* incubated for 30 days at low temperature (4 ± 1 ^{o}C) yielded 91.7 ± 0.6% of germination rate when the seeds were germinated on MS basal solid medium. Seeds stored at 60 and 90 days showed germination rates of 91.0 ± 0.8% and 77.0 ± 1.0%, respectively. Studies showed that germination rates of incubated synthetic seeds reduced as the storage period increased. Only 14.3 ± 0.7 of synthetic seeds stored for 180 days germinated.