CHAPTER - 5

Artificial Seed Technology – development of a protocol in *Arundina* and *Geodorum* – two critically endangered terrestrial orchids

5.1 INTRODUCTION

The artificial seed technology is an exciting and rapidly growing area of research in plant cell and tissue culture. Production of artificial seeds has unravelled new vistas in plant biotechnology. The commercial application of plant tissue culture technology has been successfully demonstrated. However, constraints of high labour and other expenses like hardening and delivery of tissue cultured plants remains to be overcome. In this context artificial seed technology assumes greater importance because of low cost and high volume propagation system (Rao, Ganapati, Bapat and Suprasanna,1997). The idea of artificial seeds was first conceived by T. Murashige (USA) in 1978 and researches in this area has been initiated during the last decade. It had been considered that the induction of somatic and / or pollen embryogenesis (SPE) which genetically differs from zygotic embryogenesis, is the first step and prerequisite for the production of artificial seeds.

The discovery of somatic embryogenesis (SE) and formation of pollen embryos from anther culture provide a new way for *in vitro* plant propagation. The successful induction of somatic embryogenesis has been reported in a number of plants species like cereals, millets, vegetables and other commercially important plants like coffee, tobacco, cotton and soybean (Datta 1987, Datta and Potrykus 1988, Datta and Wenzel 1987and1988, Guha and Maheshwari 1964, Guha Mukherjee 1973, Jensen 1968, Kao 1981, Mcwillium, Smith and Street 1974, Morrison and Evans 1988, Rainert 1968, Sunderland 1971, Sunderland 1974, Suprasanna *et al* 1995, Xu and Sunderland 1981,). Generally, synthetic seeds are prepared by encasing the somatic embryos within a suitable gel matrix. However, due to certain inherent problems the production rate of uniform and high quality embryoids is much low as a result of which preparation of efficient and quality seeds has not been successful excepting in a few plants like carrot, alfalfa, mulberry, ground nut, sandal wood, banana, cardamon, eucalyptus, barley and rice (Bapat and Rao 1987, 1988 and1990, Datta and Potrykus 1989, Ganapati *et al* 1992 and 1994, Huang and Cheng 1990, Kao 1981, Kitto and Janick 1985, Machi 1992, Rao *et al* 1993, Redenbough *et al* 1986, Suprasanna *et al* 1994, Wadhwa, Verma and Singh 1989).

Recent advances in this area have revealed that besides SE axillary buds, adventitious buds, shoot tips and other tissues have also been used for encapsulation (Bapat and Rao 1988 and 1990, Ganapati *et al* 1990 and 1994, Redenbough 1993,). Encapsulation of these vegetative propagules as well as the other tissues has become popular with the investigators as a simple way of handling cells and tissues and protecting them against strains and strong external gradients and as an efficient and novel delivery system (Datta and Potrykus 1989, Datta, Kanjilal and De Sarker 1999, Redenbough *et al* 1992, Singh 1991). The main advantage of the use of vegetative propagules is that it may act as a direct way to field transfer of tissue cultured plants by eliminating the tissue hardening steps. Use of these organs and tissues for encapsulation is more important for those plants like orchids where somatic embryogenesis is yet to be induced successfully.

During the last decade the encapsulation methodology has been met with success in a few plants (Redenbough *et al* 1986), most of which are non - orchid angiosperms. Orchids, the most precious and costly ornamentals are one of the few flowering plants to be propagated *in vitro* through seed germination and tissue culture.

Orchid seeds are unique in many respects. They are extremely small in size and produced in high numbers, lacking well organised embryo and have scanty / non functional or no endosperm at all and no root initials. Moreover, they need specific mycorrhizal asociation for natural germination.

The most sensational development in orchid tissue culture has been the use of *Cymbidium* shoot meristem by Morel (1960), which revolutionised the orchid industry and triggered global expansion of using tissue culture for rapid propagation of other ornamentals as well. This has been of special importance for orchids as their genotypes are highly heterozygous and vegetative propagation is an extremely slow process.

Till date, the production of synthetic or artificial seeds by encapsulating SE has not been amenable to use in case of orchids which without producing SE, gives rise to protocorm like bodies (PLBs) in culture offering a highly potential method for clonal propagation. The dust like seeds on inoculation on nutrient media produce yellow pin head like bodies which on further growth turn green. These green pin head like structures are called PLBs.

These PLBs are very fragile and need continuous supply of nutrients for complete differentiation, thus making handling and transportation difficult and expensive (Withner 1942). On appropriate balancing of auxin-cytokinine ratio, regeneration of plantlets from PLBs occur. The number of PLBs can be greatly increased in geometric progression by cutting and subculturing them repeatedly. Uniformly grown PLBs were encapsulated in the present study for production of artificial seeds.

Various stages of encapsulation has been studied in details by Redenbough et al (Redenbough *et al* 1988). One of the most important steps is the selection of suitable encapsulating agent. A number of such agents like agar, agarose, alginate, carragenan, gelrite and polyacrylamide have been used for encapsulation (Kitto and Janick, 1985). Recently nitrocellulose and ethylocellulose have also been used for encapsulation (Wadhwa, Verma and Singh 1989). However, the present investigations on *Arundina graminifolia* (Don) Hochr. and *Geodorum densiflorum* (Lam) Schltr. as well as those on a few other angiosperms suggest that the most suitable encapsulating agent for orchid PLBs has been sodium alginate due to its solubility in room temperature and formation of completely permeable gel with calcium chloride ($CaCl_2$. 2 H_2O). Uptill now, synthetic seed production by encapsulation has been successful in a few orchids such as *Dendrobium wardianum, Phaius tankervillae Spathoglottis plicata* etc.(Malemngaba *et al* 1996, Nayak *et al* 1998, Sharma Tandon and Kumar 1992, Singh 1991). The present investigation on synthetic seed production in two terrestrial and endangered orchid species namely, *Arundina graminifolia* and *Geodorum densiflorum* will be further addition to synthetic produced in orchids.

Further developmental phases in artificial seed technology has been germination of encapsulated entities followed by growth and differentiation. The primary goal of artificial seed system is the recovery of whole plant under *in vitro* and green house / field conditions. The conversion frequency under the above conditions widely varies from plant to plant. Though the frequency of plantlet regeneration from artificial seeds is reportedly high under *in vitro* condition, meagre records are available on *in vivo* conversiopn of artificial seeds under natural or green house condition. In sandal wood and alfalfa the conversion frequency varies from 17-50% (Fizi *et al* 1989 and 1992, Francis, Bapat and Rao 1992).

Orchids are yet to be explored for achieving optimal *in vivo* regeneration. The present attempt in this direction in orchids *A. graminifolia* and *G. densiflorum* is the first success story of developing a protocol for direct transfer of encapsulated seeds from laboratory to land thus eliminating the transplantation and tissue hardening steps. The advantage of this technology includes rapid and large scale multiplication, minimal labour and low cost of propagation. With the provision of supplementing the the encapsulating matrix by growth regulators, pesticides, antifungal and antibacterial agents, tailored field specific plantable units for desired crops /ornamentals can be obtained. Besides, artificial seeds would be useful material for production of desired elite genotypes as well as conservation of threatened germplasm.

5.2 MATERIALS AND METHODS

Plant material

Two taxa included in the present investigation are *Arundina graminifolia*, and *Geodorum densiflorum* . Collection of plant meterials and their maintenance have been described in Chapter I.

Seed germination and PLB production

All the technical steps including collection of capsules from experimental garden upto the formation of PLB remained same as has been described in Chapter I.

Preparation of sodium alginate solution

3.0 gm of sodium alginate (Sigma- AR Grade) was taken and mixed with 100 ml of liquid, modified VW medium (mentioned in Table1.2.2) by hand shaking. The alginate- nutrient gel thus prepared was labelled as Na- alginate-VW solution and used fresh.

Similarly, 3.0 gm of sodium alginate was mixed with 100 ml of liqued modified KnC medium, labelled (mentioned in Table 1.2.2) as Na- alginate-KnC solution and used fresh.

Preparation 50 mM calcium chloride ($CaCl_2.2H_2O$) solution

2.205 gm of $Cacl_2$, $2H_2O$ was weighed and dissolved in 300 ml of double distilled water. The solution was autoclaved, cooled and stored under refrigeration.

Preparation of PLBs prior to encapsulation

Preparation of artificial seeds by encapsulation in Na - alginate gel

PLBs measuring 2 - 4 mm in length were taken out from the culture tubes and kept on a sterile petridish. Clumps of PLBs were separeted by using sterilized forceps. Callus tissues appearing near the base of PLB were removed. PLBs were washed in liquid VW (for *A. graminifolia*) and KnC (for *G. densiflorum*).

The PLBs were then mixed with their respective alginate solution and dropped individually on the autoclaved 100 mM $CaCl_2$ solution . Calcium alginate beads were formed around PLBs within 10-15 min at 27°C on a rotary shaker moving at 80 rpm.

Encapsulated PLBs were taken out by decanting off the $CaCl_2$ solution and washed twice with sterilized distilled water to remove excess $CaCl_2$ solution. The encapsulated PLBs were then surface dried with the help of a sterilized blotting paper. Some freshly encapsulated PLBs of each of two taxa were inoculated in their respective suitable media. Amount of organic additives and growth regulators were same as used during seed germination. 150 encapsulated PLBs of each taxon were kept on a sterile petridish, sealed with parafilm and stored at 4°C in a deep freeze . These were taken out at regular intervals of 30 days and inoculated to observe their viability and regeneration capacity. Non-encapsulated PLBs were also kept at 4°C in a sealed sterile petridish. Some encapsulated PLBs were kept at room temperature (25±2°C). Observations were made at regular intervals. The whole process is depicted in the following flow chart.

Preparation of artificial seeds for *in vivo* germination

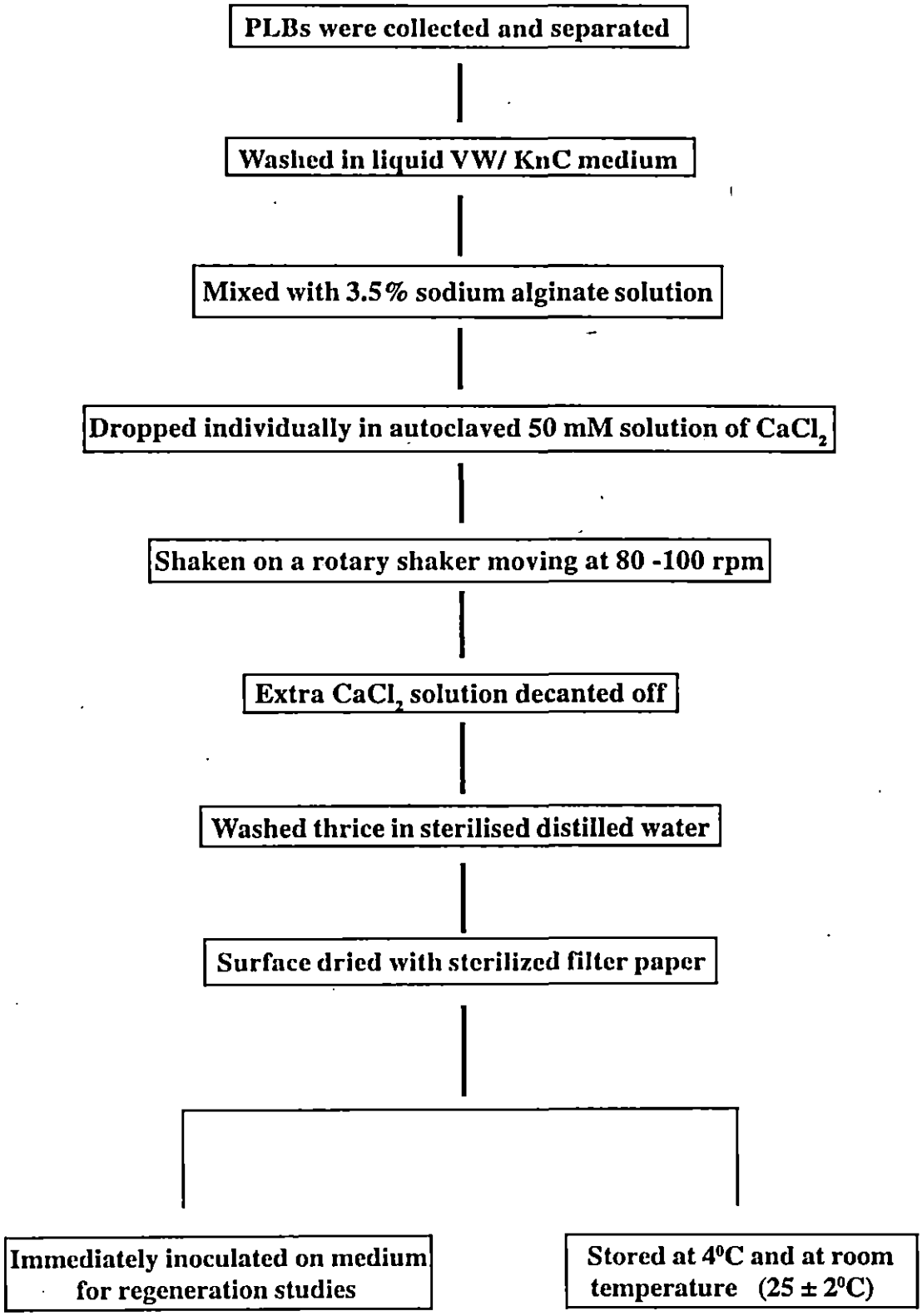
Incorporation of Fungicide and $NaHCO_3$ in nutrient- medium gel

Fungicide Bavastin was incorporated in the nutrient gel at a concentration of 4 mgl^{-1} by mixing it with alginate nutrient solution. Sodium bicarbonate was used as food preservative and incorporated in the same way. The PLBs were washed with sterilized double distilled water and surface dried with sterile filter paper and kept in a petridish.

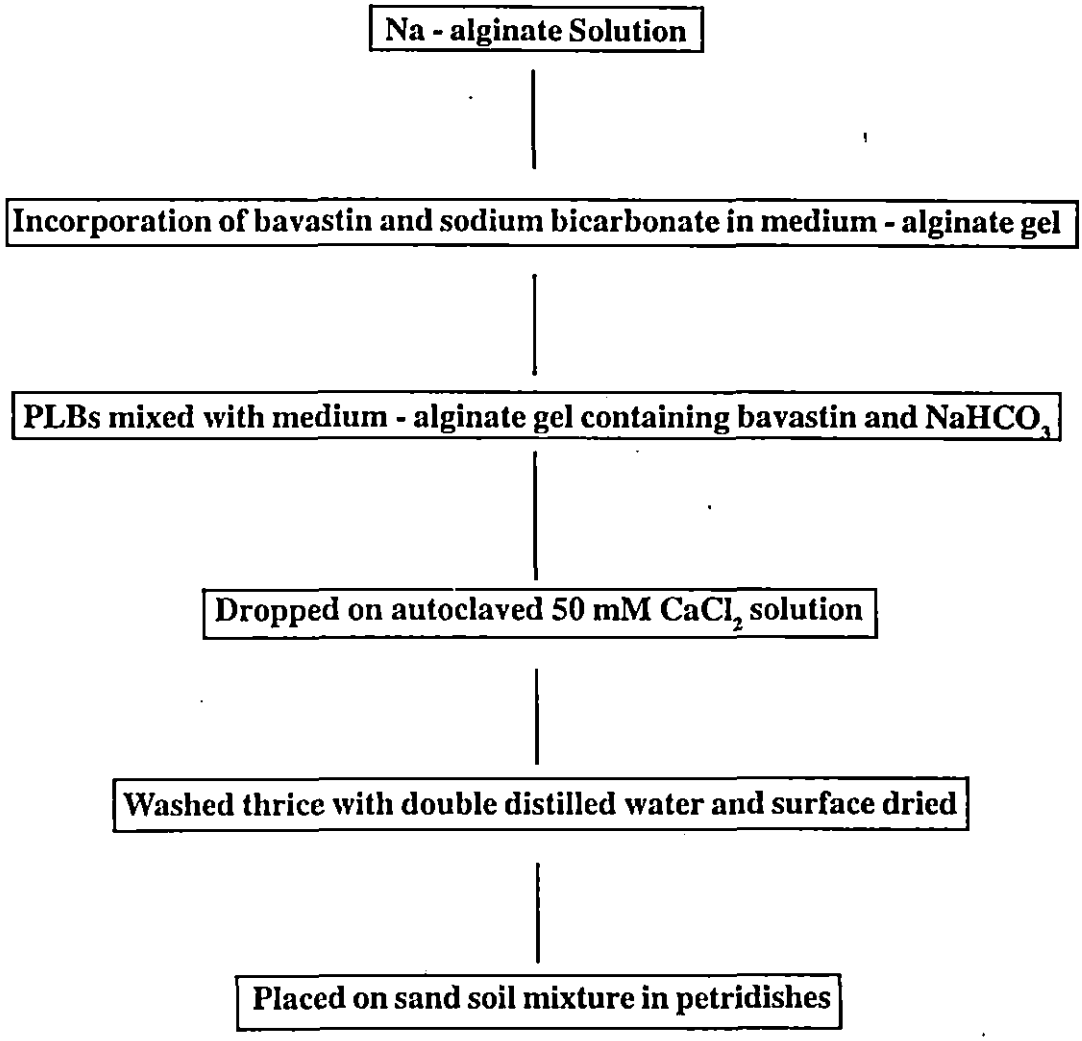
Preparation of soil

Sand and garden soil were taken and mixed at a ratio of 1:1. The mixed soil was then autoclaved and kept in petrideshes.

Flow chart showing the important steps involved in artificial seed preparation.



Flow chart showing the incorporation of fungicide bavastin and food preservative($NaHCO_3$) in medium - alginate gel.



Transfer of artificial seeds to sand - soil mixture

25 encapsulated PLBs were placed on each petridish, filled with autoclaved sand soil mixture. Distilled water was sprayed on the soil sand mixture and on theinner surface of the petrideshes. The Petridishes were covered with the lid to maintain the requird humidity and kept in the labroratory at 25 ± 2°C for 16 hr photoperiod from cool white light from fluorescent tubes giving 1000 lux at culture level. For the first 7-10 days sterilized distilled water was sprayed at regular intervals to keep the soil moist. After 10 days the lid of petridishes were removed and tap water was sprayed in the soil instead of distilled water.

5.3 OBSERVATION

Freshly encapsulated PLBs of *A.graminifolia* (Plate 15) showed induction of growth after 10-12 days following inoculation on modified VW medium supplemented with coconut milk(CM 15% v/v). The time requirement for *G. densiflorum* PLBs (Plate 16) to show growth symptom was 15-21 days when inoculated on KnC medium supplemented with CM(15%), peptone ($1gml^{-1}$), BAP ($2\ mgl^{-1}$) and NAA ($1mg\ l^{-1}$). In both the cases the PLBs first increaseed in size and started to regenerate within the alginate coat. High percentage of *in vitro* regeneration was recorded in both the cases. 94% and 88% germination and regeneration was noted in *A. graminifolia* and *G. densiflorum* respectively (Tables 5.3.1 and 5.3.2). The PLBs on growth and regeneration emerge through the matrix by rupturing the alginate coat and establish contact with the medium.These PLBs were then subcultured to their respective media and within 10-12 weeks well developed plantlets of *A. graminifolia* and *G. densiflorum* were obtained (Plate 15 and 16). As the PLBs of *A. graminifolia* and *G. densiflorum* were randomly selected for encapsulation , the time requirement for the PLBs to emerge through the matrix had been variable.

Regeneration percentage of stored encapsulated PLBs at different temperature and for different periods was recorded and shown in Tables 5.3.1 and 5.3.2 . It has been noted that the germination and regeneration percentage of *A. graminifolia* and *G. densiflorum* was almost arround 94 and 88 respectively upto 120 days and then decreased gradually with further increase in storage period. However, a sharp decrease was noted after 150 days and afterwards. After 180 days, the germination and regeneration percentage was recorded as 68 and 71 in *A. garminifolia* and *G. densiflorum* respectively.

Encapsulated PLBs stored at room temperature ($25 \pm 2^{0}C$) always showed a lower percentage of viability and regeneration in comparison to those stored at low temperature ($4^{0}C$). After 120 days only 49 and 44 percent artificial seeds were germinated in *A. graminifolia* and *G. densiflorum* respectively when stored at room temperature (Tables 5.3.1 and 5.3.2). However, after 180 days only 18% and 20% germination was achieved in *A. graminifolia* and *G. densiflorum.*

Non- encapsulated PLBs stored both at $4^{0}C$ and at room temperature showed no viability or regeneration after 30 days and died within a week following inoculation.

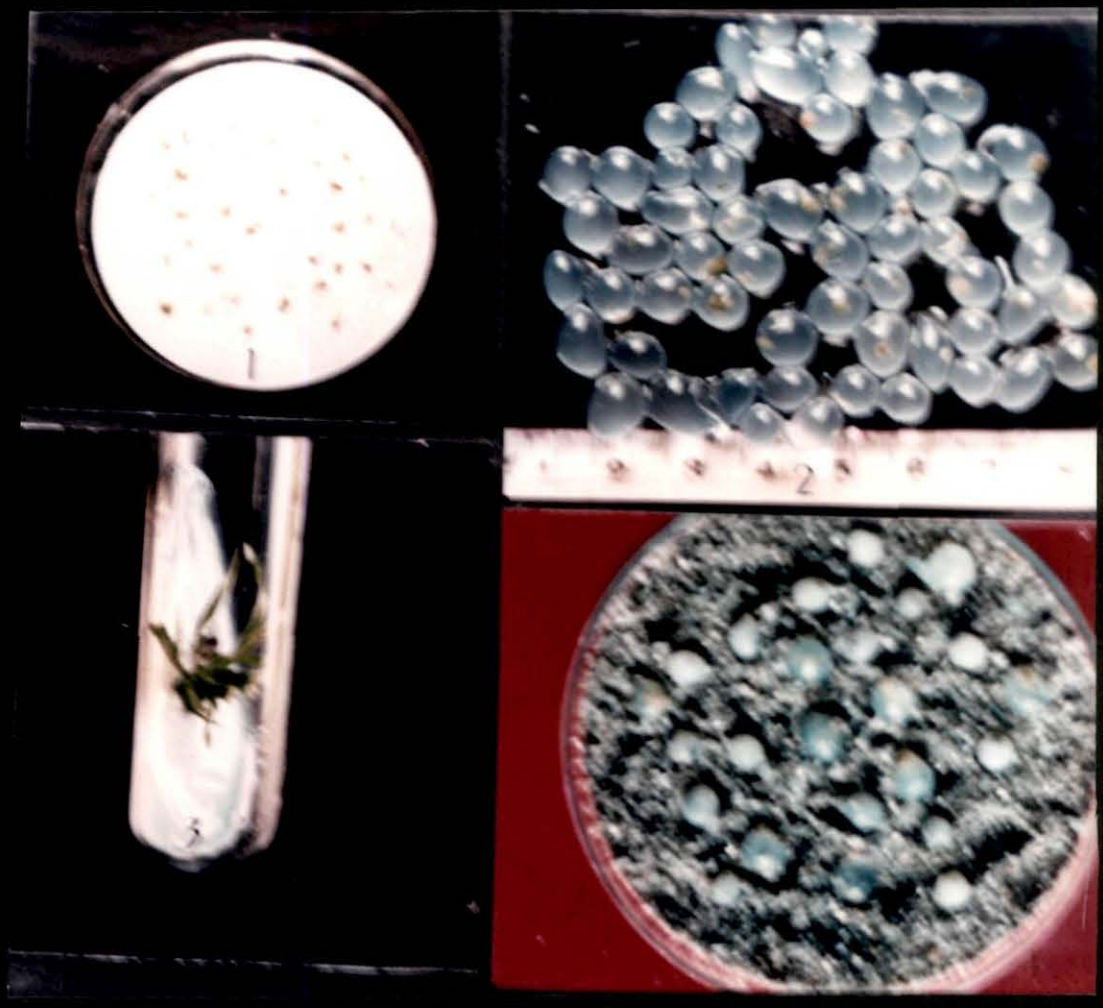


Plate 15

Table 5.3.1 Viability frequencies of the encapsulated PLBs of *A. graminifolia* at different temperature for different periods. Values are mean of 25 encapsulated PLBs in 10 replicates.

Types of Seeds	Storage Temperature (°C)	Storage Time (Days)	Regeneration Percentage ± SE
Encapsulated	No storage	No storage	94.0 ± 3.00
,,	4	**30**	93.0 ± 1.00
,,	RT	**30**	80.0**±0.90
,,	4	**60**	92.0 ±0.60
,,	RT	**60**	71.0***±0.50
,,	4	**90**	90.0* ± 1.00
,,	RT	90	62.0***± 0.90
,,	4	120	93.0 ± 0.70
,,	RT	120	49.0***±0 .30
,,	4	150	84.0 **± 1.70
,,	RT	150	37.0*** ± 0.90
,,	4	180	68.0***± 1.00
,,	RT	180	18.0*** ± 0.70
Non- encapsulated	4	**30**	00.0
,,	RT	**30**	00.0

Germination percentage of artificial seeds followed by asterisks in each treatment within the same column are significantly different from control (artificial seeds without storage), using Student's t-test at * 5% level, ** 1% level and *** 0.1% level. RT represents room temperature (25 ± 2°C).

Table 5.3.2 Viability frequencies of the encapsulated PLBs of *G. densiflorum* at different temperature for different periods. Values are mean of 25 encapsulated PLBs in 10 replicates.

Types of Seeds	Storage Temperature (°C)	Storage Time (Days)	Regeneration Percentage ± SE
Encapsulated	No storage	No storage	88.0 ± 1.05
„	4	30	87.0**±0.80
„	RT	30	72.0***±0.70
„	4	60	86.0* ±1.00
„	RT	60	64.5**± 1.28
„	4	90	87.0* ± 1.00
„	RT	90	58.0***±0.90
„	4	120	86.0* ± 1.15
„	RT	120	44.0***± 1.96
„	4	150	78.0**± 0.36
„	RT	150	36.0*** ± 1.66
„	4	180	71***± 0.80
„	RT	180	20.0*** ± 1.04
Non- encapsulated	4	30	00.0
„	RT	30	00.0

Germination percentage of artificial seeds followed by asterisks in each treatment within the same column are significantly different from control (artificial seeds without storage), using Student's t-test at * 5% level, ** 1% level and *** 0.1% level. RT represents room temperature (25 ± 2°C)

Table 5.3.3 Effects of different concentrations of sodium bi carbonate on *in vivo* germination of *A. graminifolia* and *G. densiflorum*. Each set consists of 25 encapsulated PLBs and had 10 replicates.

Concentration of sodium bi carbonate(mg l^{-1})	Concentration of bavastin (mg l^{-1})	Regeneration Percentage ± SE	
		A. graminifolia	*G. densiflorum*
0	4	10.00 ± 0.70	6.00 ± 0.30
5	4	16.00* ±1.00	12.00* ±1.08
10	4	18.00*± 0.40	20.00**±1.70
15	4	26.00**± 0.90	24.00**±1.28
20	4	41.00***±1.00	28.00**±1.00
25	4	34.00 **±0.20	22.00*±0.60
30	4	28.00* ± 0.80	20.0**± 0.70
40	4	20.00*± 0.40	18.00* ± 0.80

Germination percentage followed by asterisks in each treatment within the same column are significantly different from control (artificial seeds without sodium bicarbonate), using Student's t-test at *.5% level, ** 1% level and ***0.1% level.

Plate 16

The encapsulated PLBs containing fungicide and food preservative (sodium bicarbonate) when transferred directly to soil did not show any contamination upto 8-10 weeks. It was observed that higher concentrations of bavastin than 4 mg l^{-1} retards the growth of the PLBs within the alginate coat and lower concentrations than the same was not enough to check contamination. 4mg l^{-1} bavastin was proved optimum and hence used in all the cases. With the varying concentrations of sodium bicarbonate used, the optimum frequency of germination was noted in 20 mgl^{-1} in both the cases (Table 5.3.3). The encapsulated PLBs without any sodium bicarbonate did not show any gemrination or regeneration and the PLBs died due to dessication of alginate matrix. Gradual decrease in sodium bicarbonate level from optimum concentration results in a decrease in germination percentage. An increase by 5mgl^{-1} from 15mg-20mg triggered a significant increase in the *in vivo* germination/regeneration frequency (Table 5.3.3). At 20mgl^{-1} concentration of sodium bicarbonate, 41% and 28% germination was recorded in *A. graminifloia* and *G. densiflorum* respectively. At lower concentrations of sodium bicarbonate although some percentage of germination was noted but the continued growth of PLBs ultimately ceased due to dessication of the alginate matrix. Germination manifested as growth and differentiationof PLBs, was followed by ultimate emergence through the matrix. After emergence the PLBs established contact with the soil. Finally these started to grow on soil. At this stage the lids of the petridishes were removed and the emerged plantlets were allowed to grow in the culture room for further two weeks following which they were transferred into pots containing a mixture of brik chips, coal chips , sand and garden soil at a ratio of1: 1: 1: 1.

5.4 DISCUSSION

The present investigation deals with the production and regeneration of artificial seeds by encapsulating the PLBs of *A. graminifolia* and *G. densiflorum,* a pair of terrestrial and endangered orchids of North Eastern Himalayas. The successful production of artificial seeds and their *in vitro* and *in vivo* regeneration in these two orchids may be considered as a first attempt with a view to conservation of endangered orchids. Production of encapsulated PLBs and their *in vitro* regeneration has been reported in a few orchid species (Malemngaba, *et al* 1996, Sharma, Tandon and Kumar1992, Singh 1991).

The advent of artificial seed technology has revolutionised tissue culture production of ornamentals in general and orchids in particular. It has unravelled new vistas in plant biotechnology researches. Besides rapid and mass propagation of plants, it has added new dimensions to handling and transplantation, conservation and preservation of desirable and elite genotypes (Datta and Potrykus 1989, Singh 1991). Artificial seed technology has become very popular to the investigators as a low cost high volume propagation system and as a novel delivery system.

Many facets of artificial seed production by encapsulating PLBs have been extensively studied in two North Eastern Himalayan orchids namely, *Arundina graminifolia* and *Geodorum densiflorum* with an ultimate view to conservation of them. The primary goal of artificial seed technology is the recovery of whole plants from artificial seeds under *in vitro* and in field conditions. A very high percentage of *in vitro* germination and regeneration have been noted in both the taxa. Preservation of artificial seeds at 4°C reveals retention of high viability frequency even upto 120 days. High viability percentage of stored artificial seeds at 4°C in comparison to room temperature indicates the efficiency of low temperature for storage of encapsulated seeds. The retention of such a high percentage of viability may be due to the availability of nutrients within the gel matrix. Room temperature has been found unsuitable for storage of artificial seeds which finds support in earlier report (Malemngaba *et al* 1996).

Apart from supplying nutrients, the gel matrix also provides protection to the young fragile PLBs against mechanical injuries, dessiccation and strong external strains and stresses. However, after 120 days a gradual decrease in viability was noted which is followed by a sharp decrease after 150 days in both the taxa. Shortage of nutrients may be one of the possible reason for such decline in viability frequency

Germination of artificial seeds under natural condition is another important aspect of artificial seed technology because it will help to minimise the cost by eliminating repeated subculturing steps for regeneration and tissue hardening steps. Scanty informations are available on successful regeneration of plants under *in vivo* conditions (Fernendez, Bapat and Rao 1992, Fuji *et al* 1989, 1992, Ganpathi *et al* 1992 and1994, Redenbough 1991,). However, in the present investigation an encouragingly high percentage of (28-41%) germination under natural condition has been achieved following supplementation of the encapsulation matrix with suitable food preservative and fungicide.It was interesting to note that besides preventing dessication the food preservative sodium bicarbonate alone was effective in checking contamination. However, in long run the presence of a fungicide was essential to resist contamination.

The development of a protocol for the two endangered North Eastern Himalayan orchids may be an useful addition to the *in vivo* germination and regeneration of plantlets for storage and transportation of precious and costly orchid hybrids and for conservation of elite and endangered orchid germplasm.

The judicious and intelligent coupling of artificial seed technology with that of microcomputer in achieving automated encapsulation would tremendously increase the efficiency of encapsulation and production of artificial seeds and revolutionize the current concept of commercial micropropagation in the next millenium.

5.5 SUMMARY

Two endangered orchids of North Eastern Himalayan region, namely, *Arundina graminifolia* and *Geodorum densiflorum* have been used in the present part of work. The objectives of the present study have been to develop a suitable protocol for preparation of artificial seeds by encapsulating the PLBs of the mentioned orchids and their germination and regeneration under *in vitro* and soil conditions.

A systematic investigation was undertaken for the preparation of artificial seeds by encapsulating the PLBs in sodium alginate gel and their germination under different conditions. PLBs of *Arundina graminifolia* and *Geodorum densiflorum* were obtained by germinating the seeds in modified VW and KnC medium respectively. The amount of organic additives and concentrations of growth regulators were same as used during seed germination and described in Chapter I. 25 - 30 day old PLBs were selected for encapsulation. The PLBs were washed with double distilled water. 3% sodium alginate solution was prepared by dissolving it separately in liquid VW and KnC media. PLBs were mixed with nutrient-alginate gel and dropped singly on an autoclaved 50 mM solution of calcium chloride ($CaCl_2$. 2 H_2O). Calcium alginate beads were formed around the PLBs within 10-15 minuites on a rotary shaker moving at 80 rpm. Encapsulated PLBs or artificial seeds were taken out and washed with double distilled water to remove excess $CaCl_2$. 50 freshly encapsulated PLBs of each of *Arundina graminifolia* and *Geodorum densiflorum* were inoculated on modified VW and KnC medium with same concentrations of organic additives and growth regulators as used during seed germination. 150 encapsulated PLBs of each studied genera were kept on sterile petridishes sealed with parafilm and stored at 4°C. Some encapsulated PLBs were kept on sterile petridishes and stored at room temperature (25 ± 2°C). Non- encapsulated PLBs were also stored at both 4°C and at room temperature. Fungicide bavastin and various concentrations of sodium bicarbonate was incorporated into the gel matrix for *in vivo* germination of encapsulated seeds.

High percentage of *in vitro* germination of artificial seeds were noted in both the cases. 94 and 88 percent germination were observed in *Arundina graminifolia* and *Geodorum densiflorum* respectively. On germination the PLBs emerge out through the encapsulating matrix and establish contact with the medium and within 10-12 weeks following inoculation well developed plantlets were obtained. Regeneration percentage of stored encapsulated PLBs at different temperature for different periods were recorded. It has been observed that upto 120 days the germination percentage was very being around 94 and 86 percent in *Arundina graminifolia* and *Geodorum densiflorum* respectively. However, further increase in storage period resulted in gradual decrease in germination percentage and a sharp and significant decrease was noted after 150 days and afterwards. Encapsulated PLBs stored at room temperature showed comparatively low percentage of viability. This indicates low temperature is more suitable for storage of artificial seeds. Non-encapsulated PLBs stored both at 4^0C and at room temperature did not show any germination after 30 days and died within a week following inoculation.

The encapsulated PLBs containing fungicides and different concentrations of sodium bicarbonate did not show any contamination upto ten weeks following their inoculation in soil. With varying concentrations of sodium bicarbonate used, optimum germination percentage was noted in 20 mgl-1 concentration in both the cases. 41% and 28% germination was noted in *Arundina graminifolia* and *Geodorum densiflorum* respectively. The encapsulated PLBs without any sodium bicarbonate did not show any germination. An increase by 5 mg in sodium bicarbonate concentration from 15-20 mg triggered a significant increase in germination percentage. Although a low percentage of germination was noted in lower concentrations of sodium bicarbonate, the continued growth of PLBs ceased due to dessication. Though sodium bicarbonate alone can check the contamination the presence of a fungicide was required in long run.

The present investigation clearly suggests the efficiency and suitability of the developed protocol. The advent of artificial seed technology has revolutionised

the tissue culture industry. The present investigation on two endangered orchids will add a new dimension towards this direction.The developed method, has been proved useful in encapsulating the PLBs, which serves as a means of conserving tissue and transporting it to different places as well. Moreover, with this technology it will be possible to eliminate the most difficult steps of hardening.