Chapter-3

MODEL SYSTEM: *Kalanchoe*

INTRODUCTION:

Natural seeds are usually genetically heterogeneous and much time is needed to produce seeds that are heterogeneous and much time is needed to produce seeds that are homogeneous in terms of their genes. However, if developing embryos obtained by somatic embryogenesis are used directly for the propagation of plants or are encapsulated in suitable material that promotes germination, plant clones with the same genes as the mother plant can be obtained simply and standard laborious breeding procedures can be avoided. This synthetic seed technology is one of the most developing fields of plant science in the last decade. They are generally prepared by encasing the somatic embryos obtained from tissue cultures in a protective jelly capsule, which is prepared by desiccating the somatic embryos with or without coating. Encapsulated embryos can be stored for the preservation of valuable germplasm.

Murashige (1978) defined synthetic seed as an ***encapsulated single somatic embryo***- a clonal product that could be handled and used as a real seed for transport, storage and sowing and that would grow eventually either *in vivo* or *ex vitro*, into a plantlet (***conversion***). This definition limited the synseed production to the use of somatic embryo i.e. a bipolar propagule generated through somatic embryogenesis and that could be contained in a capsule, which would allow easy manipulation and establishment of a seedling. Bapat *et al* (1987) proposed the making of synseeds through the encapsulation of *in vitro* derived propagules different from somatic embryos, especially non-embryogenic structures and used the axillary buds of *Morus indica* for encapsulation.

Broadly, now the synseeds can be defined as artificially encapsulated somatic embryos, shoots or other tissues, which can be used for sowing under *in vitro* or *ex vitro* conditions (Aitken-Christie *et al* 1995). Hence, the concept of synseed can be extended to any type of vegetative propagule. But then it should be emphasized that the propagule must be able to grow into a plantlet after sowing. Under this category, series of reports have appeared in the literature (Standardi & Piccioni 1997).

Table–3.1 Nonembryogenic (Unipolar) vegetative propagules used for synthetic seed production

- NUPs – natural unipolar propagules includes microbulbs, microtubers, rhizomes and corms.
- MCs – micro cuttings includes nodes with apical or axillary buds, shoots
- DPs – differentiating propagules includes immature tissues such as meristemoids, cell aggregates and primordia.

Propagule grouping and speices	**Propagule type**	**References**
NUPs		
Cymbidium giganteum	Protocorms	Corrie & Tandon 1993
Kalanchoe tubiflora	Epiphyllous buds	Palmer & Jasrai 1996
Lilium longiflorum Thumb.	Microbulbs	Piccioni *et al* 1992; Standardi *et al* 1995
Nephrolepis sp.	Rhizome fragments	Rhizome fragments
Spathoglottis plicata	Protocorms	Singh 1991
MCs		
Actinidia delicosa Liang & Fergusaon	Apical and axillary buds	Piccioni & Standardi 1995a
Betula pendula Roth	Apical and axillary buds	Piccioni & Standardi 1995a
Betula davurica	Apical and axillary buds	Lee *et al* 1990
Boehmeria nivea	Adventitious buds	Chen *et al* 1996
Cedrela odorata I	Apical and axillary buds	Maruyama *et al* 1997a, 1997b
Dioscorea spp	Axillary buds	Hasan & Takagi 1995
Ellettaria cardamomum Maton	*Ellettaria cardamomum* Maton	Ganapati *et al* 1994
Guazuma crinita Mart.	Apical and axillary buds	Maruyama *et al* 1997a, 1997b

Ipomea aquatica Forsk	Axillary buds	Tang *et al* 1994
Jacaranda mimosaefolia D. Don	Apical and axillary buds	Maruyama *et al* 1997a, 1997b
Malus spp	Apical and axillary buds	Piccioni & Standardi 1995a, Piccioni 1997
Morus alba L.	Adventitious buds	Machii 1992; Pattnaik & Chand 1996
Morus indica L.	Apical and axillary buds	Bapat *et al* 1987; Bapat & Rao 1990; Bapat 1993
Musa sp.	*Musa* sp.	Ganapati *et al* 1992; Matsumoto *et al* 1995
Olea europea L.	Apical and axillary buds	Piccioni *et al* 1996b; Micheli *et al* 1998
Rubus spp	Apical and axillary buds	Piccioni & Standardi 1995a
Valeriana wallichii DC	Apical and axillary buds	Mathur *et al* 1989
Viburnum odoratisumum	Axillary buds	Tang 1996
Zingiber officinale Rosc.	Axillary buds	Sharma *et al* 1994
Solanum tuberosum	Nodal shoot segments	Sarkar & Naik 1998
DPs:		
Armoracia rusticana	Adventitious shoot primordia	Uozumi *et al* 1994; Uozumi & Kobayashi 1995
Armoracia rusticiana	Cell aggregates	Repunte *et al* 1996
Armoracia rusticiana	Root fragments at different stages	Nakashimada *et al* 1995, 1996
Cymbidum giganteum	Protocorm like bodies	Tandon *et al* 1994
Oryza sativa L.	Shoot primordia	Yoshida 1996
Dendrobium wardianum (D. pendulum) Warner	Protocorm like bodies	Sharma *et al* 1992; Tandon *et al* 1994

Thus artificial or synthetic seeds or synseeds consists of somatic embryoids or meristemoids encased in a protective gel or a covering. These synseeds offer great advantage in the field of biotechnology, if it is scaled up and commercialized. Though, somatic embryos are most convenient and logical propagules of choice for the production of artificial seeds but then this technology can also be extended for encapsulation of axillary / apical / adventitious shoot buds etc for the production of synseeds. Encapsulation of the epiphyllous buds of *Kalanchoe tubiflora* was carried out and thus it was established as a model system for the standardization of the synseed technology.

Kalanchoe: *Kalanchoe tubiflora* var. verticillata belonging to the family crassulaceae commonly known as the **chandelier plant** is a slender, erect, succulent plant with pinkish-brown stem with many, almost cylindrical tube like, pinkish leaves bearing small buds at their tip (Fig-1 a & b) These epiphyllous buds of *K. tubiflora* offers an excellent experimental material to study synseed technology because of its following advantages.

(1) Ease of bud development: As the development of complete plantlet occurs at predetermined sites.

(2) Rapid response: The complete development of plantlets with roots occurs within ten days after isolation.

(3) Ease of experimental manipulation: The bud growth as such does not require any exogenous nutrient medium, as water is only adequate for entire experimentation.

(4) Amount of material: Availability of large number of epiphyllous buds per plant. Hence the epiphyllous buds of *K. tubiflora* were utilized for studies as a model system for encapsulation technology.

Also use of *K. tubiflora* propagules (epiphyllous buds/meristemoids) is an excellent system for classroom demonstrations and laboratory technique for synthetic seed production (Palmer & Jasrai 1996).

Fig-1 a) *Kalanchoe tubiflora* plant

b) Note the epiphyllous buds at the tip of *K. tubiflora* leaf


b
a

MATERIAL AND METHODS:

Plant material:

The epiphyllous buds of *K. tubiflora* were collected from the plants grown in the Botanical garden, M.S.University of Baroda. The buds were washed thoroughly under running tap water, followed by washing with teepol for 2 min and thereafter rinsed with distilled water. Later on they were subjected to surface sterilization with $HgCl_2$ solution (0.1 % w/v).

Encapsulation:

The epiphyllous buds were then dipped in Sodium alginate solution (2-5 %) and dropped one by one in Calcium Chloride solution (0.06 M). Surface complexation begins immediately and firm round beads are formed. During complexation reaction the metal ions are cross-linked through ionic bonds between $COOH^+$ groups present on guluronic acid molecules of the alginate. Alginates that are rich in guluronic acid, therefore allow the formation of hard and compact beads. The resultant buds each containing a single bud were kept on a gyrotatory shaker (30 min) for hardening. The $CaCl_2$ solution was subsequently decanted and the buds were washed with sterile distilled water.

For inducing dormancy in the synseeds, ABA was used in such a way that the epiphyllous buds were treated with different concentrations of ABA (0.0001–0.1 mM) and also it was added in the alginate matrix prior to encapsulation. There are two types of artificial seeds basically, hydrated and desiccated, for desiccated synseeds the resultant buds were subjected to air drying for different time intervals under sterile conditions (2-6 min)

Storage:

The resultant synseeds were transferred to small petridishes (3 cm dia) sealed with parafilm, and kept in airtight desiccator and stored at 5° C in refrigerator.

Germination:

The encapsulated synthetic seeds germinated easily on agar based semi-solid medium. Water alone was also used for the germination of epiphyllous buds when subjected to exogenous ABA treatment.

RESULTS AND DISCUSSION:

A) Effect of different concentrations of Sodium alginate on synseed formation

Encapsulation using sodium alginate is widely used for the production of synthetic seeds as it is a simple direct process that can be done using a dropper, pipette, forcep or a spatula to form alginate drops as they fall into a complexing bath ($CaCl_2$ solution) to form calcium alginate capsules or beads. Thus for encapsulation, different things like forceps, spatula, glass pipette and droppers were tried.

The shape and the coating around the bud was good and sizable when the beads were formed using droppers. Synseeds of *Asparagus cooperi* baker (Ghosh & Sen 1994), *Arachis hypogea* (Padmaja *et al* 1995) and horse radish (Shigeta & Sato 1994)were formed by dropping, the somatic embryos into a solution of $Cacl_2$ by means of a pipette. And the encapsulated matrix was good enough to provide physical coating and protection against any mechanical damage during handling. Synseeds of *Eleusine coracana* (George & Eapen 1995), *Morus indica* (Bapat & Rao 1988)& *Santalum album* (Bapat et al 1987) were formed by using forceps.

The beads were kept in calcium bath for about half an hour on the shaker and when hardness of capsule was achieved they were washed with water. The minimum complexing time in calcium chloride was 15 min. Otherwise the center of the capsule remained in liquid state.

The different percentage of sodium alginate were tried (2-5 %) with calcium chloride solution (0.06M), because it is necessary to standardize the correct concentrations of sodium alginate for the formation of quality beads.

Table–3.2: Different percentage of sodium alginate for the formation of synseeds

Concentrations of Sodium alginate (%)	Nature of beads (synseed)
2	Coating not sturdy enough, handling difficult
3	Coating not sturdy enough, need extra care
4	Sturdy beads, normal germination
5	Formed thick coating

Amongst the different concentrations of sodium alginate tried 4% proved to be optimum for coacervation with calcium chloride (0.06M) for the formation of good, sturdy beads and did not cause inhibition of germination of *K. tubiflora* buds (Table-3.2)(Fig-2 b). When the lower concentration (2-3 %) of sodium alginate tried for bead formation, the beads were not sturdy enough for handling and prone to mechanical damage during handling. Embryos of *Asparagus cooperi* that were encapsulated in 3.5 % sodium alginate showed highest conversion percentage, whereas at a higher percentage of sodium alginate 6-7 %, beads were harder which suppressed the ability of shoots and roots to emerge. Similar reports of a maximum plantlet regeneration frequency with an optimum concentrations of 3.2 % sodium alginate has been reported in alfalfa and celery (Redenbaugh et al 1986) and 3 % for *Solanum melongena* (Lakshmana Rao & Singh 1991). Somatic embryos of *Arachis hypogea* encapsulated in 2 % sodium alginate resulted in the formation of soft beads that were not suitable for handling (Padmaja et al 1995). Thus the concentration of alginic acid also influence the frequency of conversion due to the hardness factor of the beads.

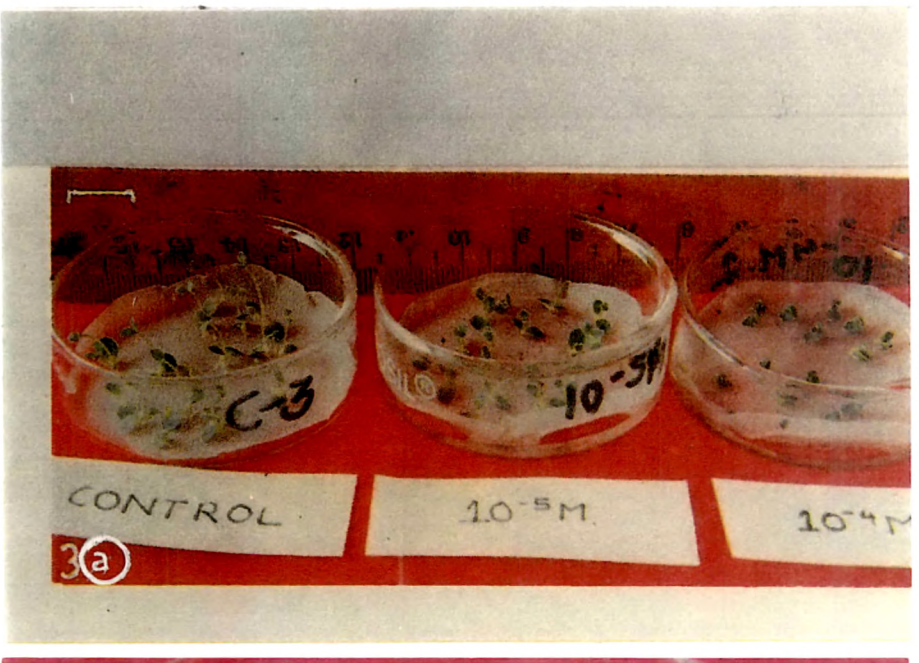
The successful use of non-embryogenic vegetative propagules like axillary buds obtained from *in-vitro* established shoot-cultures e.g. axillary buds of Mulberr-*Morus indica* (Bapat *et al* 1987), apical and axillary buds of six woody species e.g. *Actinidia deliciosa, Betula pendula, Crataegus oxyacantha, Malus sp.*, Rubber spp. and *Rubus idaeus* (Piccioni & Standardi 1995), axillary or apical shoot-tips of *Cedrela odorata* (Maroyama *et al* 1997, protocorm like bodies (PLBs) of *Spathoglottis plicata* (Singh 1991), cell aggregates (CA) derived from horseradish hairy roots (Repunte *et al* 1996), single node cuttings of potato *Solanum tuberosum* (Sarkar & Naik 1998) etc have been reported.

B) Exogenous ABA treatment to *K. tubiflora* buds

The encapsulation of epiphyllous buds of *Kalanchoe tubiflora* for standardizing a protocol for synseed technology for its applications to other plants proved to be very efficient system. The epiphyllous buds are at a stage wherein a slight trigger (their isolation from leaves) induces growth response (Jasrai *et al* 1996). Hence, when the encapsulated buds were kept for storage precocious development of bud into shoot was observed even at low temperature. Thus effect of ABA – a known growth inhibitor was studied, at different concentrations like (0.0001 mM, 0.001 mM,

Fig-2 a) Effect of Exogenous appplication of ABA on the growth of *K. tubiflora* buds

b) Emergence growth of shoot from the synseed of *K. tubiflora*


C-3
CONTROL
10^{-5}M
10^{-4}M
3(a)


(b)

0.01mM, & 0.1 mM). For exogenous treatment of ABA the *K. tubiflora* buds were kept in small petridishes with filter paper. ABA solution of different concentrations was poured in the petridishes with pipette. Thereafter they were transferred to the culture room. Amongst different concentrations of exogenous application of ABA tried 0.1 mM proved to the optimum concentration in inducing dormancy (Fig-2 a) (Table-3.3)

Table-3.3: Exogenous application of ABA to *K. tubiflora* buds

Concentrations of ABA (mM)	Germination (%)*
0.0001	100.00 ± 0.0
0.001	59.37 ± 5.98
0.01	25.00 ± 5.10
0.1	0.00 ± 0.0

* values are the percentage means of three independent experiments

Compton *et al* (1992) reported that maize somatic embryo, when pretreated with 0.1 mM ABA they remained viable over 2 week after dehydration at 70 % relative humidity. In fact, ABA is known to inhibit the growth of somatic embryos of other plants like celery (Nadal *et al* 1989), carrot (Kitto & Janick 1985) and alfalfa (Senarta *et al* 1989).

C) Encapsulation of the *K. tubiflora* buds treated exogenously with ABA

A set of exogenously treated epiphyllous buds with 0.1 mM concentration of ABA were encapsulated and were stored in small petridishes sealed with parafilm at low temperature in refrigeration. As the storage period advanced there was a corresponding decline in the germination behaviour of *Kalanchoe* synseeds (Table-3.3). The pretreated synseeds of *k. tubiflora* showed 90 % and 77.5 % germination after 10 and 20 days but thereafter they showed a decreased rate of germination, remaining viable till 90 days with 50 % germination. The treated buds of the synseeds remained viable for 3 months in storage at low temperature with 50 % survival.

Table – 3.3: Germination of encapsulated buds of *K. tubiflora* (pretreated with 0.1 mM ABA).

Days of storage	Germination (%)*#
Control	100.00 ± 0.00 [a]
0	100.00 ± 0.00 [a]
10	90.00 ± 4.08 [a]
20	77.5 ± 2.50
30	65.0 ± 2.88 [b]
40	62.5 ± 4.78 [b]
50	60.0 ± 4.08 [bc]
60	57.5 ± 2.50 [bc]
70	60.0 ± 4.08 [bc]
80	55.0 ± 2.88 [bc]
90	50.0 ± 4.08[c]

- Means within a column followed by the same superscript letter are not significantly different ($P \leq 0.05$; Duncan's multiple range test).

\# values are the mean percentage of three different experiments

Generally two broad categories of arrested growth, **quiescent** and **dormant** are distinguished for buds. Quiescence is a resting phase that can be reversed solely by addition of water. Dormancy is a form of arrested growth that requires factors in addition to water, such as cold or heat treatments, for resumption of growth to occur (Bewley & Black 1985). Reports on encapsulated microbulbs (*Lilium*) (Standardi *et al* 1994), epiphyllous buds (*Kalanchoe*) (Palmer & Jasrai 1996), or protocorms (Orchids)(Tandon *et al* 1994) do not usually describe any particular induction treatment required by the explants in order to be able to develop into plantles, while, in contrast, they sometimes need precocious growth inhibition with abscisic acid (ABA) or other treatments.

D) Encapsulation of *K.tubiflora* buds along with ABA (O.1 mM) in the alginate matrix

Another set of epiphyllous buds was encapsulated along with ABA (0.1mM) in the alginate matrix to study its effect to continuous exposure to the growth inhibitor.

Initially for about 40 days dormancy was induced and not much precocious development of buds was observed, and when kept for germination it showed aroun 57 % germination but after about 70 days the germination declined tremendously and the buds showed senescence probably because of continuous exposure to ABA in the matrix (Table-3.4).

Table –3.4: Effect of ABA in the synseeds of *K. tubiflora* in the alginate matrix on germination

Days of Storage	Germination (%)*
Control	100.00 ± 00
0	100.00 ± 00
10	67.50 ± 4.78
20	70.00 ± 4.08
30	65.00 ± 2.88
40	57.50 ± 2.5
50	47.50 ± 4.7
60	40.00 ± 4.08
70	22.50 ± 2.5
80	7.50 ± 2.5
90	No growth

* values are the mean percentage of three indepentent experiments.

The encapsulation of the buds does not prevent the precocious development of the buds. When water was used instead of MS medium, the synseeds showed better development than control i.e., the non-encapsulated buds in *Morus alba* (1992) Machii. Thus encapsulation itself stimulated the growth of adventitious buds in Mulberry. The conversion rates reported for micropropagated cuttings are often very high, also after cold storage, when sowing is done on agar-solidified medium (Maruyama *et al* 1997b, Capuano *et al* 1998). They are generally much lower on a soil like substratum or in *ex vitro* conditions, unless appropriate treatments are performed to the capsules ie, addition of an antimicrobial substances or the physical chemical status of the substratum is consistent with hydration and nutrient needs of the encapsulated microcuttings (Bapat & Rao1990; Machii & Yamanouchi 1993).

E) Effect of air-drying on encapsulated buds of *K. tubiflora*

To induce desiccation and to increase the viability of artificial seeds the resultant synseeds were air dried under sterile conditions ie exposed to laminar airflow for different time intervals ie 2-6 minutes (Table-3.5). At low exposure to the air-flow the synseeds remained sticky, and difficulty to handle, whereas increased exposure (5 min) to desiccation treatment increased not only the viability of the encapsulated buds but were easily handled.

Table 3.5: Effect of air-drying on synseeds of *K. tubiflora*

Minutes	No. of Days 30	No. of Days 60	No. of Days 90	No. of Days 120
Control	90.02 ± 4.08	70.00 ± 3.3	50.00 ± 1.7	35.00 ± 1.5
2	80.00 ± 3.0	55.50 ± 2.8	32.40 ±1.8	29.60 ± 0.8
3	77.50 ± 2.5	57.00 ± 2.8	40.00 ± 2.0	12.60 ± 0.8
4	60.00 ± 2.5	60.00 ± 2.5	48.50 ± 2.8	11.30 ± 0.6
5	57.50 ± 2.5	62.50 ± 3.0	50.00 ± 2.8	10.00 ± 0.6
6	50.00 ± 2.0	58.00 ± 2.5	21.60 ± 1.6	00.00 ± 0.0

* values are the means of three independent experiments

Abscisic acid has been shown to induce desiccation tolerance in orthodox somatic embryo (Carman, 1988; Welbaum *et al* 1990). However viviparous seeds that are mutants of plants that normally produce orthodox seeds produce very little ABA, and have been demonstrated to be unable to tolerate desiccation (Karssen *et al* 1983 Koornheef *et al* 1989). Etienne *et al* (1993) reported that *Hevea brasiliensis* recalcitrant somatic embryos acquired desiccation tolerance as a result of treatment with high levels of ABA.

The desiccation treatment not only inhibited the precocious development of buds but also increased the viability of synseeds and the artificial seeds of *K. tubiflora* remained viable for about 4 months with 10 % germination after exposure to air drying under sterile conditions in laminar airflow. This treatment of successful exposure to desiccation reported in carrot somatic embryos where they were dried to 17 % moisture and stored for 4 months in the dry state at room temperature, approximately

67 % of the dried embryos converted into plants (Huang *et al* 1994). Desiccation tolerance of broccoli microspore derived embryos was induced by exogenous application of ABA. A one-day exposure to ABA had the similar effect on the induction of desiccation tolerance as a 7-day treatment. The dried somatic embryos maintained their ability of plant conversion even after three months of storage under room conditions (Takahata *et al* 1993).