Chapter-1

CONCEPTUALISATION

INTRODUCTION:

The much-awaited new millennium has started with the same old unaccomplished problems and to make the matter worse now a days there is severe drought in many parts of the world. As if this is not enough in India the population has crossed above one billion. Thus there has to be a scale up of annual food production from present 1800 million metric tons to almost 3000 million metric tons. Most of the people in the developing countries rely only on agriculture as their occupation. Under the adverse conditions and the limited cultivated area the agriculturists have to be equipped in this modern age with something that will help them to boost up the productivity of the major food and economic crops.

Over the years due to extensive investigation into problems related to basic and applied aspects of plants, the field of applied and experimental botany has advanced rapidly. Factors responsible for growth metabolism, differentiation and morphogenesis of plants have been explored in depth because of the vast knowledge of tissue culture. Biotechnology is a key technology for future developments wherein plant biotechnology is the application of current scientific methods and techniques to agriculture, horticulture, forestry, energy, food, pharmaceuticals, cosmetic and service industries. The rapid development of biotechnology in recent years has contributed greatly to the advancement of plant sciences.

Plant tissue culture comprises a set of *in-vitro* techniques/methods and strategies that are the part of group of technologies called Plant biotechnology. Tissue culture is a process whereby a small piece of living tissue ie explants are isolated from an organism and grown aseptically for indefinite periods on a semi-defined or defined nutrient medium (Ignacimuthu 1996).

The applications of biotechnology to crop production are:

1. The combination of meristem / shoot tip culture and heat treatment for the production of virus free seedlings.
2. Application of techniques of tissue culture and suspension culture for the preservation of plant germplasms.

3. The method to overcome cross incompatibility between distantly related species by fertilization and embryo rescue for the production of disease and insect resistant cultivars.
4. The improvement of breeding efficiency by the utilization of haploid plants from anther culture.
5. The utilization of liquid suspension culture for mass propagation, mutation breeding and the production of artificial seeds and secondary products.
6. The production of somatic hybrids and material for genetic engineering studies by protoplast culture and fusion (Lin *et al* 1996).

The Tissue culture system has also been exploited to create genetic variability so as to increase the number of desirable germplasms available to the plant breeder.

The Tissue culture protocols are available for most crop species, although continued optimization is still required for many crops. The Tissue culture techniques in combination with molecular techniques have been successfully used to incorporate specific traits through gene transfer. Large-scale micropropagation laboratories are providing millions of plants for commercial ornamental market and the agricultural, clonally propagated crop market. With selected laboratory material typically taking one or more decades to reach the commercial market through plant breeding, this modern technology can be expected to have an ever increasing impact on plant improvement as the approach in the new millenium (Browne & Thorpe 1995).

Historical resume:

Schwann in 1839 proposed the cell theory, which stated that each living cell of an organism, if provided a proper environment would be capable of independent development. This theory gave birth to the concept of totipotency. Trecul in 1853 observed callus formation in a number of decorticated trees. Vochting (1878) inspired by Schwann's cell theory, performed same basic experiments and stated that in every plant fragment lies the elements from which, by isolating the fragments under proper external conditions, the whole body can be built up. Wiesner (1884) proposed a general theory that suggested the existence of organ-forming substances distributed in a polar fashion. Rechinger (1893) observed the formation of new buds & callus from explants isolated from buds, roots and stems. Klercker (1892) isolated protoplasts

Kuster achieved the first fusion in 1909, a noted cytologist who established that some salt solutions facilitated this process.

It was Gottlieb Haberlandt (1902) who conceived the idea of culturing isolated plant cells in the nutrient solutions. Based on cell theory, he assumed that there were no limitations of divisibility, therefore he started with isolated mesophyll cells using Knop's nutrient solution, sucrose, aspargine and peptone. The cells survived 3-4 weeks but without cell division. Habertandt predicted that one-day ***artificial embryos*** would be successfully cultivated from vegetative cells, he developed the concept of *in-vitro* cell culture and thus regarded as the father of tissue culture.

Nobecourt a French plant pathologist, announced simultaneously in 1939 with White and Gautheret, the possibility of cultivating plant tissues for unlimited period, which was possible with the use of the discovery of indole-3-acetic acid (IAA) by Went (1932).

Morel in 1950 obtained the indefinite growth of monocotyledonous tissue such as *Gladiolus*, *Iris* and lily on the medium, which was enriched with natural extracts like coconut milk (CM) and yeast extract (YE). White, an American Scientist reported for the first time successful and continous culture of tomato root tips and obtained indefinite growth of roots. Steward was one of the pioneers of plant tissue culture. He used CM for the first time in plant tissue culture and obtained vigorous proliferation of carrot explants. Steward and Shantz in 1955 were the pioneers in establishing cell cultures (single cells and clumps) in liquid medium.

Another landmark was the induction of somatic embryogenesis in carrot cell suspension cultures and production of complete plants (Reinert & Steward, 1958). This has not only opened new avenues of micropropagation of plant species at an enormous rapid rate but also completely established the totipotency of the plant cells so as to produce whole new plants under favourable conditions of nutrients and plant growth regulators.

Somatic embryogenesis:

Somatic embryogenesis is a multiplication process where haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages (without the fusion of gametes). Plants are unique in their ability to produce somatic embryos. Somatic embryos are structurally similar to zygotic embryos found in seeds, they have characteristic feature which includes the ability to grow into complete plant without separated shoot development and rooting phases.

On the onset of the 19th century Haberlandt claimed ***one should successfully grow artificial embryos from vegetative cells*** based on the cell theory of Schwann & Schleiden (1939). However it was not until 1958 that somatic embryos were for the first time detected and recognized as *in-vitro* cultures independently by Reinert and Steward in cultures derived from multi-cellular explants of *Daucus carota*. At present somatic embryogenesis is a widespread phenomenon.

Sharp *et al* (1980) defined somatic embryogenesis as the process wherein a bipolar structure arises through a series of stages characteristic for zygotic embryo development and having no vascular connection with the parental tissues. Somatic embryogenesis get initiated when a single cell or small group of cells initiates the developmental pathway normally followed only by the pre-dormant embryo within the seed.

Plants like legumes, cereals, herbaceous plants and woody perennials were considered difficult to propagate using somatic embryogenesis 10-15 years ago (Ammirato 1983). Now the successful embryogenesis for legumes, cereals and herbaceous plants has been reported whereas woody horticultural & forestry species continue to represent a major challenge. Although somatic embryogenesis of many tree species has been reported, the defined pathway generally involves either embryogenic or juvenile explants (Litz & Gray 1992). Somatic embryogenesis is initiated by either of the two cell types -

(a) *Pre-embryogenic Determined Cells* (PEDCs) which are already determined for the embryogenic pathway and await only the synthesis of an inducer (or removal of an

inhibitor) to resume independent mitotic divisions and express their embryogenic potential

(b) *Induced Embryogenic Determined Cells* (IEDCs) which require redetermination to the embryogenic state, generally by exposure to specific growth regulators (Sharp *et al* 1980).

PEDCs are found in embryogenic tissue, including the scutellum of cereals, in certain tissues of young *in-vitro* grown plantlets, and the nucellus and embryo sac within the ovules of mature plants. Somatic embryogenesis via the route of IEDCs is more difficult to induce, since the starting material consists of differentiated vegetative cells that must undergo major epigenetic changes to initiate somatic embryo production. IEDCs occur in callus cultures, particularly after treatment with powerful synthetic auxins such as 2,4-D. Once the embryogenic state has been induced, however, there appears to be no fundamental difference between IEDCs and PEDCs. Depending on culture conditions the full embryogenic pathway may be followed to produce a complete plant directly or it may be ***short-circuited*** by escape of individual cells or small cell group to reinitiate embryogenesis independently. Escape of cells from co-ordinated development may give further embryoids, or, if occurring continuously, may give rise to nodular embryonic callus (Williams 1987).

The potential application of somatic embryogenesis in plant production depended to a large extent on whether proliferation is directly from PEDCs without cellular destabilization and redifferentiation, or, whether an intervening destabilized callus phase is required for redetermination to produce IEDCs. Direct somatic embryogenesis from PEDCs appears to produce relatively uniform clonal material, whereas the indirect pathway involving IEDCs tends to generate a higher frequency of somaclonal variants.

Cells capable of direct somatic embryogenesis are physiologically similar to those in zygotic embryos. They are frequently found either in tissue before the onset of embryogenesis (ie in the flower organ) or in the developing zygotic embryo. In these cells the genes necessary for zygotic embryogenesis occurs, that can be seen as a ***memory*** of pathway either previously or just after the zygotic embryogenesis. Cells of tissue which are in time or space more diverged from zygotic embryo explants need a

greater amount of reprogramming of previously active developmental pathways before they reach the embryogenic ground state (Sharp *et al* 1980, Raemakers *et al* 1995). The ability to express embryogenesis is often restricted to narrow developmental phase. In many species immature zygotic embryos have the ability to express somatic embryogenesis while mature zygotic embryos do not possess it (Finer 1987).

Many researchers in the area of forest biotechnology regard somatic embrogenesis as the *in-vitro* system of choice for mass propagation of superior and genetically engineered forest tree genotypes (Gupta *et al* 1991). For both coniferous and hardwood species, somatic embrogenesis has a number of advantages over other micropropagation techniques, namely axillary shoot multiplication and adventitious shoot production.

The advantages, most commonly well appreciated, include very high multiplication rates and the potentials for scale up in liquid culture (ie bioreactors) and for direct delivery to the greenhouse or field as artificial seeds. Such features make it likely that clonal propagules produced via somatic embryogenesis will have significantly lower per unit costs than those produced using other microprogation system due to lower labor costs including to make excellent target material for gene transfer via ***Agrobacterium*** based ***Ti*** plasmid-mediated and biolistic transformation (Mc Granahan *et al* 1988, Wilde *et al* 1992, Ellis *et al* 1993). Thus, it is widely believed that embryogenic cultures will eventually be employed for commercial-scale production of clonal propagules for forest trees (Merkle 1995) and other economically important plants.

Dramatic progress in induction of somatic embryogenesis and production of somatic embryo derived plantlets of hardwoods has been made in the past decade, notable among them are mango (Jana *et al* 1994), *Teminalia* (Kumari *et al* 1998), Quercus (Kim *et al* 19894), Pomegranate (Bhansali 1990), *Prunus* (De March *et al* 1993), *Citrus* (Cabasson 1997) etc. But it is puzzling to note that not a single forest tree species is currently being propagated commercially using embryogenic cultures though direct and indirect somatic embryogenesis has now been shown to be possible in a number of plant families.

Applications of Somatic embryogenesis:

Somatic embryogenesis offers a number of following potential applications –

1. Since both, the growth of embryogenic cells and subsequent development of somatic embryos can be carried out in a liquid medium, it is possible to create large scale mechanized or automated culture system which are capable of producing propagules (somatic embryos) repetitively with low inputs.
2. Somatic embryos form IEDCs show high frequency of somaclonal variation. As the somatic embryos mostly developed form single cell or a group of cells formed form the division of a single cell, the regenerants will be somaclonal variant (Ignacimuthu 1996) which can be screened for specific selection procedure.
3. Nucellar embryos being free of virus can be used for raising virus free plants.
4. For many of the tree species, somatic embryogenesis from nucellar cells may offer the only rapid means of obtaining juvenile plants equivalent to seedlings with the parental genotype.
5. Somatic embryos of selected elite parents are potentially convenient organs for cryopreservation and germplasm storage.
6. Artificial seeds, consisting of somatic embryos enclosed in a protective coating, have been proposed as a low-cost-high-volume propagation system. The advantages of artificial seeds are the production of many somatic embryos and the use of conventional seed handling techniques including seed delivery. The objective is to produce clonal seeds at a cost comparable to true seeds.
7. Embroygenic callus, suspension cultures and somatic embryos have been employed as a source of protoplast isolation for a range of species. Cells and tissue in these systems have demonstrated the potentiality to regenerate in culture and therefore, yields protoplasts that are capable of forming whole plants.
8. Mature trees can be rejuvenalised for propagation purposes by somatic embryogenesis.
9. With somatic embryos, in principle, discrete propagules are produced which posses the developmental program to grow into a complete plant without additional shooting and / or rooting steps which are necessary in micropropagation systems.
10. Labor costs can further be reduced if somatic embryogenesis is conducted in liquid medium in appropriate-size bioreactors.

Artificial or Synthetic seeds:

The concept of synthetic seeds (synseeds, artificial seeds or somatic seeds) was first proposed by Toshibo Murashige (1977) at the symposium on ***Tissue Culture for Horticultural Purposes***, held at Belgium, as ***an encapsulated single somatic embryo*** ie a clonal product that could be handled and used as a real seed for easy transport, storage and sowing so that such propagules would eventually grow either *in-vivo* or *ex-vitro*, into a plantlet. In simple terms it means a somatic embryo entrapped in a biodegradable synthetic polymer coating that acts as an artificial seed coat.

Natural seed may be defined as a ripened ovule consisting of an embryo and its coat. Anatomically, a seed consists of embryo, seed coats derived from integument and nucellus. The normal seeds, contain materials in their endosperm which it utilizes during the process of its germination. Hence endosperm may contain variety of stored material depending upon the plant species such as starch, oils, proteins, etc. For any structure that is to be considered as ***seed*** should contain an embryo like entity, and so majority of research efforts in the area of artificial seed production have been pursued in system where prolific somatic embryogenesis has been documented (Redenbaugh *et al* 1986).

Apart from somatic embryos, artificial or synthetic seeds may also consist of meristemoids or propagules encased in a protective gel or covering. The artificial or synthetic seed therefore can be considered equivalent to true seeds wherein the gel acts both as an artificial endosperm (a reservoir of nutrients) and as artificial seed coat protecting the embryo (or even meristemoid) from mechanical damage during handling.

Advantages of artificial seeds:

Artificial seed technology offers following advantages –

1) Economy of space, nutrients, labor and time leading to better cost-benefit ratio.
2) It is an effective delivery system that provides viable alternative to presently employed high cost incurring vegetative propagation techniques.

3) Direct sowing in the field is possible with a seed size propagule. This would bypass the acclimatization step required during transplantation in a conventional micropropagation procedure.
4) To handle a small propagule (few mm) rather than a large plantlet (few cm or inch) provides tremendous flexibility in transfer of technology from lab to land. The size of the propagule is also crucial for storage, shipping and planting.
5) The encapsulation coating has the potential to hold and deliver beneficial supplements such as growth promoters, fertilizers, nutrients, pesticides and symbiotic microorganisms. These could be precisely placed around individual plants.
6) Artificial seeds have a particular relevance in the propagation of hand-pollinated hybrids, elite germplasm and genetically engineered hybrids with sterility or unstable genotype complications.
7) By inducing dormancy through appropriate manipulations in the coating matrix or by following the usual storage techniques, artificial seeds can provide unique opportunities for the conservation of endangered or threatened germplasm.
8) As an academic tool, artificial seed offers comparative and for better understanding of zygotic embryogeny role of endosperm during embryo conversion and seed coat formation.

Synthetic seed productions have been well documented in many plant speices belonging to diverse families.

Table-1: Various reports on synseeds of different plants:

Plant	Authors	Year	Germination
Apium graveolens (Celery)	Cao *et al*	1989	*In-vitro* and in soil
Apium graveolens Var. rapaceum	Li *et al*	1990	*In-vitro* and in soil
Arachis hypogea (Ground-nut)	Padmaja *et al*	1995	33.3 %
Armoracia lapathifolia (horseradish)	Shigeta &Sato	1994	Encapsulated 74 % Nonencapsulated 54 %
Asparagus cooperi	Ghosh & Sen	1994	8.3% after storage for 90 days at 2^0 C
Brassica olearacea	Takahata *et al*	1993	27-48 % desiccated
Brassica napus	Tay *et al*	1993	100 %
Carica papaya	Castillo *et al*	1998	77.5 %
Carica papaya	Ye *et al*	1993	80 % under sterile conditions 72 % in field
Camellia japonica	Janeiro *et al*	1997	40 % after 30 days 30 % after 60 days
Copties chinensis	Ke *et al*	1990	-----
Daucus carota	Kitto & Janick	1985	40 % survival
Dendroacalamus strictus	Mukunthakumar & Mathur	1992	45 % in soil 96 % *in-vitro*
Eleusine coracana Gaertn.	George & Eapen	1995	100 % after 2 days no germination after 14 days
Ipomea batatas	Schultheis *et al*	1994	-------
Medicago sativa	McKersie *et al*	1990	-------
Pelargonium horteum	Gill *et al*	1994	-------
Pelargonium X hortorum *Pelargonium X domesticum*	Marsolais *et al*	1990	18 of 30 cultivar synseeds germinated after rehydration. 80 – 100 % of somatic embryos germinated after rehydration
Trigonella ruthenica	Zhow *et al*	1996	-------
Zea mays	Compton *et al*	1992	Embryos greater than 5mm survived desiccation

Types of Artificial seeds:

Artificial or Synthetic seeds or Synseeds are basically of two types 1) Hydrated and 2) Desiccated, which includes both coated and uncoated somatic embryos.

Hydrated Synseeds consist of somatic propagule (embryo or shoot bud) individually entrapped in a hydrogel. The use of hydrated coatings become necessary because of poor desiccation tolerance of somatic embryos. Hydrogel encapsulated somatic embryos have conversion frequencies equal to noncoated embryos when planted *in-vitro*. Microspore derived embryos of barley (*Hordeum vulgare*) when encapsulated in hydrated gel showed 80 % germination compared to uncoated embryos, which germinated at 62 % (Takahata *et al* 1993).

The most widely used method for production of synseeds is hydrogel encapsulation using sodium alginate. This is a simple, direct process that can be done using a dropper, spatula to insert somatic embryos singly into the drops as they fall into a complexing bath to form calcium alginate capsules or beads that are approximately 2-4 mm in diameter. Also the embryos and sodium alginate can be mixed together and then dropped in the complexing bath.

Sodium alginate will complex when mixed with di and trivalent metal cations to form calcium alginate via formation of ionic bonds between carboxylic acid groups on the guluronic acid molecules of the alginate. Alginates rich in guluronic acid form harder capsules than alginates with high levels of mannuronic acid. Sodium alginate will stabilize and remain stable at room temperature (25^0 C). It does not require heat to produce a gel, but begins complexing immediately when brought into contact with metal cations. The hardness of the capsule is a function of the guluronic : mannuronic acids ratio, the cation and the complexing time. Minimum complexing time in calcium chloride is 15 mins. Otherwise the center of the capsule will still be liquid. Viscosity of the sodium alginate and the inside diameter of the nozzle to form the drops can control the size of the capsule. A capsule hardness of 0.5 to 2.0 kg breaking pressure per capsule was found to allow germination while providing sufficient integrity so that the capsules can be handled on a routine basis without breakage.

Alginic acid is a linear, 1,4-linked co-polymer of β-D mannuronic acid and its C_5 epimer, α–L-guluronic acid. Most commercial alginates are extracted with sodium hydroxide from giant brown seaweeds such as *Macrocystis pyrifera* (Sigma Chemicals Co. and Kelco) and *Laminaria hyperberea* (Proton Als). When the monovalent ion of sodium is replaced by divalent ions like Ca^{2+} ionic cross-linking among the carboxylic acid groups occurs, and the polysaccharide molecules form a polymeric network. Gels with the highest mechanical strength, lowest shrinkage, best stability towards monovalent cations, and highest porosity were especially made from alginate with a content of L-guluronic acid higher than 70 %. Due to its simplicity sodium alginate is a choice hydrogel because of its features like:

1) Easy complexation with calcium chloride through an ion exchange reaction.
2) Biologically non-damaging
3) biodegradable
4) easy and universal availability and
5) low-price.

Other hydrogels have been tested for synthetic seed production like Carrageenan and locust bean gum. Carrageenan used by itself had too high a gelling temperature which killed the embryos, whereas addition of locust bean gum to the carrageenan sufficiently reduced the gelling temperature so that the embryos survived. Redenbaugh *et al* (1996), Nigam *et al* (1988) used various methods for producing hydrogel capsules, alginate/poly-l-lysine, chitosan and agarose. But they were not as useful as alginate for encapsulation. Using agar and gelrite, synseeds can also be formed by lowering the gel temperature, using a moulding process. Propagules can be mixed with gelrite (0.25 %) and poured into flat bottom microtiter wells, lowering the temperature can produce the capsules.

In order to maintain an adequate level of hydration, the encapsulated seeds must be coated with a water-impermeable hydrophobic coating. Such coatings should also reduce tackiness of the encapsulated embryos. The coatings must adhere to the hydrogel encapsulation material and maintain its stability at room temperature in capped bottles or sealed containers. The coating should be biodegradable, and if

solvents are used to coat the capsules, the procedures and solvents must be harmless to the embryos. These conditions suggest that the coatings, which prevent evaporation of water from foods or used in water proofing compositions would be effective coatings for somatic embryos. Potential polymers useful as binders are ethylene vinyl acetate copolymers (Elvax 310,410 and 4260) and cellulose derivatives such as ethyl cellulose and ethylhydroxyethyl cellulose (EHEC). The Elvax products are ethylene vinyl acetate (EVAc) copolymers manufactured by Dupont (Wilmington, DE). Due to the acid content of Elvax, this polymer shows adhesion to polar, nonporous materials. Adhesion of the hydrophobic coating to the hydrogel capsule material is an important requirement for successful development of coated synthetic seeds. Ethyl cellulose (EC) can be combined with hydrophobic waxes and polymers to produce water proof coatings, however the coated capsules tend to stick to each other and water retention of the capsules is not as good as those coated with Elvax. Ethylhydroxyethyl cellulosic derivative is soluble in some aliphatic solvents and compatible with waxes. It shows good adhesion to the hydrogel seed capsules however capsules coated with EHEC show only slight stability improvement relative to uncoated capsules. The samples lost from 6 to 12 % of water content per day.

Desiccated somatic embryos are produced by mixing somatic propagules with 2.5 % polyoxyethylene glycol (Polyox WSR-N 750) and then dispensing 0.2 ml of this mixture on a teflon surface in form of wafers. These are then allowed to dry under sterile conditions. The wafers are then placed on suitable substratum, allowed to rehydrate and score for embryo conversion. Embryos that remain green are considered to have survived, dead embryos quickly turned dark brown.

Kitto and Janick (1982) reported for the first time the formation of polyox wafers using embryogenic suspension of carrot containing isolated cells, cell clumps, callus and embryos of varying maturity, where only 3 % of the coated embryos survived desiccation. Gray *et al* (1987) reported that in Orchard grass (*Dactylis glomerata*) somatic embryos could be desiccated to 13 % moisture level and they could be stored for 21 days at 23^0 C in the form of polyoxyethylene glycol wafers. The conversion frequency of the coated somatic embryos, however decreased from an initial 32 % without storage to 8 % after 7 days and 4 % after 21 days of storage because during

dehydration somatic embryos decreased in size, become brittle and their outer cell walls collapsed. Thus the polyox coating seems to provide embryo protection by preventing lethal embryo desiccation. In Celery embryo encapsulation with polyox at 70 % RH, the synthetic seeds survived 7 to 9 days of desiccation and they produced normal plants (Kim & Janick 1989).