Chapter 2

Literature review

Rice is considered in Gramineae family, genus *Oryza* and is found in nearly every continent. There are 23 rice species which are devided into 2 types: 21 species of wild rice and 2 species of cultivated rice which are *Oryza sativa* L. and *Oryza glaberrima* Steud. They can be planted in a wide range of water – soil regimes, from deeply flooded land to dry, hilly slopes. Moreover, most of them are diploid basic, in other words, they have 12 chromosome pairs (2n=24) whereas the minority which is in genus *Oryza* is tetraploid or has 24 chromosome pairs (2n=48). Rice generally has almost 100 percent self-pollination because its pedicels are perfect flowers. The self-pollination is the effect of non-flowering, even though less frequent, so that the pollen fertilize with the pistil in its flower. The anther may break, the pollen will therefore fertilize with pistil before blooming. The result of self-pollinationed in flowers are called “autogamy”.

2.1 Crop improvement :

Genetic improvement of crops or economically important plants by conventional methods of breeding typically needs several years to develop a new variety. Conventional rice breeding which depends on natural somaclonal variation has many restrictions, one of them is that it requires extra time and effort in

developing new species. Moreover, interspecific hybridization such as cross wild species chromosome number aberration with cultivated species might be impossible because of incompatibility and possibly some disadvantagous characteristics from wild rice in selected hybrid species. Thus, biotechnology and genetic engineering approach could be the best solution for those problems. Biotechnology applied in rice breeding is categorized into 3 procedures: tissue culture, genetic engineering and DNA-based marker with Marker Aided Selection. Hybridization is the first step, which involves cross-pollination and role of anther comes into picture. The inherent limitations with hybridization process, is the segregation of F1 offspring, which produces genetic variability within the F2 population. In other words, crosses between distantly related species can bring together novel gene combinations. However, the hybrid offspring can be few in number, genetically unstable and require years of further selection and screening before any agronomic trait is brought to commercial use (Giri and Giri, 2007).

2.2 Hybrid rice :

Hybrid rice is rice that has been created by crossing two different parental strains. Such crosses generally result in an F1 generation that is more robust than either of the parental strains. The improved qualities of the F1 generation is referred to as "hybrid vigour" or "heterosis". The hybrid vigour may result in superior agronomic qualities such as higher yield, stronger resistance to diseases, more efficient use of soil nutrients, and better weed control. Hybrid vigour and other

superior qualities arising from crossing genetically different plants has been well known and used by traditional crop breeders for decades (Cheng *et al*., 2005).

In the past, the production of hybrid rice strains was limited by rice's inherent propensity to self-pollinate. In 1974, Chinese scientists overcame this when they developed the first generation of hybrid rice using a three-line hybrid system based on cytoplasmic male sterile (CMS) lines and hybrid combinations. In 1996, an even more efficient second generation of hybrid rice was developed based on photoperiod-sensitive genetic male sterility (PGMS) lines.

Traditional rice production (i.e., non hybrid rice) relies on rice varieties. A rice variety is a rice line that is a group of rice plants distiguished by common characteristics of significance to agriculture and often has been assigned a commercial name. When rice is produced from a variety, a single line is planted and it fertilizes by self-pollination. When a rice variety is reproduced, it retains its distiguishing characteristics, and farmers can keep seeds for replanting next season. In contrast, hybrid rice is the product of a cross between two distinct rice lines, and due to the difficulty of making hybrids, they are generally only produced by seed companies. Farmers do not save seeds for replanting because self-fertilization will result in genetic segregation of traits. Therefore, farmers need to buy new hybrid seeds every year. This may produce an economic hardship for the farmer, who has to balance the benefits of hybrid vigour with the annual cost of purchasing new seeds (Yuan, 2002).

2.3 Haploid and doubled haploid plants production :

Plants with monoploid may occur during anther culture of diploid plants from meiosis cell division process. Monoploid plants (1n) from anther culture could perform somaclonal variation characteristics controlled by recessive genes from the haploid. The culture of polyploid pollen, such as tetraploid (2n=4x) was discovered, according to the previous studies, that induced plantlets whose chromosome number reduced to half (n=2x) could not reveal somatical variation controlled by recessive genes due to the suppressing chromosomes. The study by Schaeffer *et al*., (1979) suggested the success of haploid plants production *in vitro* complete in at least 79 species. The production of haploid plants and doubled haploid plants from anther culture offers a rapid achievement of homozygous lines for early release of new crop varieties. Many desirable traits such as high grain weight, disease resistance, dwarf plant type and abiotic stress tolerance were introgressed into rice breeding population by culturing of anthers. Unfortunately, low percentages of both callus induction and plant regeneration are the principal constraints in establishing successful anther culture in some rice varieties especially in *indica* rice since these critical culturing responses are genotype dependent (Roy and Mandal, 2005).

Rice has become one of the few crops in which anther culture can be rapidly applied in breeding programs (Chen *et al*., 1991). Extent of success of haploid induction depends on a number of factors that include genotype, developmental stage, cultivated conditions of plants, components of culture medium, pre treatments etc. (Shih-Wei and Zhi-Hong, 1992; Bala-chandran *et al*., 1999; Wang *et al*., 2000; Datta, 2005). Exploitation of anther culture technique in breeding and genetics research is limited due to anther culture of *indica* and *indica* x *japonica* rice very low

regeneration frequency of anthers of rice in general, and *indica* cultivars in particular (Balachandran *et al*., 1999). As Zhang (1989) stated, there is relatively over dominance for culture ability and therefore making the cross combinations among the parents with different culture abilities can overcome the culture difficulties. Research efforts on the enhancement of response to anther culture have been confined mostly on manipulation of callus induction and plant regeneration protocols (Balachandran *et al*., 1999). According to Reddy *et al*. (1985), the application of N6 medium to *indica* rice was found to be limited although it is quite suitable for japonica rice. Therefore attempts were made to modify this medium in order to improve its suitability.

2.4 *In vitro* techniques :

Developments of the *in vitro* techniques offer possibilities of introducing into plants variability that could be utilized for crop improvement. Haploids with their unique genomic constitution have potential for accelerating the production of homozygous new varieties. The production of rice haploids and subsequent homozygous diploid plants by *in vitro* anther culture has dramatically advanced in the last 15 years. The application of this technique for improvement of rice varieties has still been hindered by the difficulty of inducing morphogenesis, either directly from the cultured anthers or indirectly from callus derived from microspores. Also the callus produced frequently loses. The plant regeneration ability with time in culture makes studies on selection of callus mutants difficult when longer periods of the *in vitro* culture are required. Another difficulty arises when during culturing *in vitro* all rice varieties do not respond equally in producing callus and in regenerating plants.

This could be due to genetic or environmental characteristics of different varieties (Ozawa *et al.,* 2003). There are several factors, such as genotype, physiological state of the donor plant, physiological stage of the microspores, culture medium, growth regulators, sucrose and shock pre-treatments that affect the response of anther culture for producing androgenic callus and plant regeneration. Among the external factors the exogenously applied hormones, mainly auxins, such as 2,4-dichlorophenoxy acetic acid (2,4-D), play a critical role in the reactivation of the cell cycle and in the initiation of the embryo formation. Application of high concentrations of 2,4-D in the culture medium itself is a stress signal since embryogenic induction requires the use of physiological auxin concentrations that inhibit the callus growth (Dudits *et al*., 1991). It was shown that anther culture of F1 hybrids leads to fixation of gene combinations. Otherwise, it would be impossible to isolate from a segregating population for developing homozygous lines as well as heterotic F1 hybrids (Hu and Zeng, 1984).

Hu and Zeng (1984) compare three breeding methods in rice, with anther culture, bulk method and pedigree method. Among the three methods, anther culture was ideal for developing homozygous lines in the shortest period of time (in first generation). The doubled haploids (DH) produced from the F_1 by anther culture increase selection efficiency, especially when dominance variation is significant. In conventional breeding, early generation lines exhibit variation due to both additive and dominance effects whereas DH lines derived from anther culture of an F1 will show only additive variance; therefore, high heritability can be expected due to the elimination of dominance effects. Thus, compared to an F2 population, fewer DH plants are needed to select desirable recombinants.

2.4.1 Anther and pollen culture :

The basis of pollen and anther culture is that on an appropriate medium the pollen microspores of some plant species can be induced to give rise to vegetative cells, instead of pollen grains. This change from a normal sexual gametophytic pattern of development into a vegetative (sporophytic) pattern, appears to be initiated in an early phase of the cell cycle when transcription of genes concerned with gametophytic development is blocked and genes concerned with sporophytic development are activated (Sunderland and Dunwell, 1977). The result is that in place of pollen with the capacity to produce gametes and a pollen tube, microspores are produced capable of forming haploid pro-embryos (somatic embryos formed directly from the microspores), or callus tissue. The formation of plants from pollen microspores in this way is sometimes called androgenesis. Haploid plants are more readily regenerated by culturing microspores within anthers than by culturing isolated pollen. The presence of the anther wall provides a stimulus to sporophytic development. The nature of the stimulus is not known but it may be nutritional and/or hormonal. Embryogenesis has only been induced from isolated pollen of a very small number of plants.

Androgenesis in plants provides an understanding of the biological basis of single cell microspore embryogenesis to the production of a doubled haploid plant. This system provides an excellent opportunity to shorten the breeding cycle for rapid production of doubled haploids and fix agronomic traits (Suriyan *et al*., 2009). There are many factors that can affect the success of anther culture, such as the maturity of the donor plant (Afza *et al*., 2000; Jacquard *et al*., 2006), panicle pretreatment (Trejo-Tapia *et al*., 2002), microspore developmental stages (Afza *et al*., 2000; Cha-Um *et al*., 2009). In almost all species genotype no doubt is a deciding factor in achieving

success in anther culture response (Ramakrishnan *et al*., 2005; He *et al*., 2006). The genetic makeup of *indic*a subspecies makes them recalcitrant to *in vitro* anther culture.

The culture of immature anthers and pollen grains is done so as to induce the pollen grains to develop into multicellular forms, particularly into embryos, with half the normal number of chromosomes for the species. When such haploid embryos are treated with chromosome doubling agents, e.g. colchicines, their normal chromosome number is restored (and thus their fertility) and the achieved plants are pure lines. Pure line formation in a natural tendency for self-fertilizing species and can be obtained with cross-fertilizing species only after repeated inbreeding for 10 or more generations.

So far, the induction of haploid plant formation from anther and pollen cultures has been successful mainly with naturally self-fertilizing species and thus, on chromosome doubling, are in theory very similar if not identical with the parents. However, by first crossing many lines from a self-fertilizing species, new combinations of genes are formed and haploid plants produced by anther culture from such crosses can be an extremely valuable and quick way of obtaining pure lines of these new combinations.

2.4.1.1 The benefit of anther culture :

The benefit of anther culture is distinct in accelerating the selection process, because of high degree of homozygous doubled-haploid (DH) plants or pure lines which can be obtained at first generation in a single step. Selection process can be done earlier since anther culture technique allows early expression of recessive genes

and increase selection efficiency as the number of plants required to obtain the desired recombinants are less than the conventional breeding (Dewi and Purwoko, 2001). The objective of anther culture or pollen culture is to produce haploids plants for homozygous diploid by using colchicines to induce diploidization within one generation whereas the conventional method takes 5-6 generations of selfing. Somaclonal variation from tissue culture is possible but often recessive, therefore, it takes time for variable characteristics to be noticeable due to cells' conditions with diploid or polyploidy. Plants with monoploid may be produced from diploid' s anther culture from meiosis. Monoploid reveals somaclonal variation controlled by recessive genes as it is monoploid (Schaeffer *et al.,* 1979). The doubled haploid plants resulting from anther culture are homozygous and breed true. Superior genotypes identified provide an additional opportunity for useful genetic recombination to occur (Singsit *et al*., 1990; Bjornstad *et al*., 1993). These homozygous lines can be brought into a breeding programme for the genetic improvement of crop plants.

Anther culture involves the culture of immature pollen or microspores within the anther *in vitro*. Pollen is haploid and the cells produced from pollen or microspores during culture are haploid as well, so we expect haploid plants. Haploid plants are regenerated from anther in culture *in vitro* by two pathways. Directly, plants regenerate from anther possibly by somatic embryogenesis (androgenesis), or develop into callus tissue, which in turn is induced to regenerate plants under the influence of growth regulators added to the culture medium. Haploid plants are distinguishable from diploids with particular reference to rice crop.

2.4.1.2 Modes of androgenesis (Giri and Giri, 2007) **:**

There are two modes of androgenesis, the direct and indirect.

- Direct androgenesis

In this, the microspore behaves like zygote and undergoes various stages of embryogeny simulating those *in vivo*. The embryos mostly at the globular stage are released from the exine and develop further. Finally, the cotyledons unfold and the plantlets emerge from the anthers.

- Indirect androgenesis

In contrast to the direct androgenesis, the microspores instead of undergoing embryogenesis, divide a few times to form callus which bursts through the anther wall. This mode of development is quite common and is usually caused by a complex media and in cases where the polarity seems to be disturbed. The callus either differentiates to form embryo or roots or shoots on the same medium or it has to be transferred to another medium. The callus derived plants are mostly undesirable as they exhibit genetic variations and polysomy.

2.4.1.3 Factors affect on androgenesis :

Some of the factors are extremely critical for the success of anther culture. The factors such as the genotype of the plant as source of anthers, developmental stage of the pollen and composition of the nutrient media and pretreatment of the anthers prior to *in vitro* culture are important. Improving survival of pollen in *in vitro* culture increases the chance of inducing androgenesis. The cold shock given flowers before

culturing the anthers to induce the pollen toward androgenesis also affects the viability of the pollen in culture. The favorable effect of cold pretreatment of flower buds or inflorescences on induction frequency has been reportd for rice and other species. In anther culture of hsien rice, the frequency of green plantlet induction has been rather low. But when panicles were pretreated at 9-11 ºC for 14 days, induction frequency in 4 materials averaged 7.6 % (number of green plantlets/100 anthers inoculated). Shan-You No. 2 pretreated for 20 days reached an induction frequency of 31.2%. As many as 86 microspores within 1 anther developed into multicellular pollen (Zhou and Cheng, 1981). In serial culture experiments on keng rice, the best results were obtained from pretreatment at 10 ºC for 20 days. The induction frequency of callus was 15 times more than in the control (Chen *et. al.*, 1980). Haploid plants can be produced either by a complete anther culture or by isolated microspore culture. Anther culture of rice was first reported in the year 1968 (Niizeki and Oono, 1968). Several new rice cultivars have also been developed from anther culture (Zhang and Chu, 1986)

2.4.1.4 Stage of the microspores and stress pretreatment :

From dissolution of the tetrad to the first mitosis in the microsporogenesis of rice, three major stages, namely, early, mid and late uninucleate can be differentiated based on the size of the vacuole and the position of the nucleus in the microspore. Microspores at the mid-late to late uninucleate stage have the highest embryogenic ability in barley (Hoekstra *et al*., 1992).

For most species a suitable stage for the induction of androgenesis lies between just before or just after pollen mitosis. During this period the cells are

noncommittal in their developmental potential. After the first mitotic division the cytoplasm gets populated with gametophytic information and it gradually becomes irreversibly programmed to form male gametophyte. This stage may be reached within 24 hrs after pollen mitosis (Scott *et al*., 1991).

A variety of stresses applied during the labile developmental period of the pollen grain can mask the gametophytic programme and induce the expression of sporophytic-specific genes and, thus, induce the pollen to switchover from gametophytic mode to sporophytic mode of development. In some cases the stress of excising the anthers and placing them under culture conditions is adequate to bring about this shift (Sunderland, 1974). In others, treatments such as temperature shocks (high or low), high osmolarity and starving the pollen grains of sugar or other nutrients are required to induce or promote the induction of androgenesis.

In order to switch the gametophytic programme of the microspore development to the sporophytic pathway, stress pretreatment of the anthers is needed. Cold-shock pretreatment has showed to be very effective for induction of androgenesis. The spikes are placed in a plate at 4 ºC for 14-28 days at relative high humidity in the dark (Huang and Sunderland, 1982). Another stress pretreatment which is widely used is the carbohydrate starvation. This system is based in the substitution of a metabolizable sugar (sucrose, maltose) by a non-metabolizable sugar like manitol. The anthers are cultured for 4 days at 24 ºC on 0.3 M liquid mannitol (Roberts-Oehlschlager and Dunwell, 1990).

2.4.2 Callus cultures:

Callus is a coherent and amorphous tissue, formed when plant cells multiply in a disorganised way. It is often induced in or upon parts of an intact plant by wounding, by the presence of insects or microorganisms, or as a result of stress. Callus can be initiated *in vitro* by placing small pieces of the whole plant (explants) onto a growth-supporting medium under sterile conditions. Under the stimulus of endogenous growth regulators or growth regulating chemicals added to the medium, the metabolism of cells, which were in a quiescent state, is changed, and they begin active division. During this process, cell differentiation and specialisation, which may have been occurring in the intact plant, are reversed, and the explant gives rise to new tissue, which is composed of meristematic and unspecialised cell types. During dedifferentiation, storage products typically found in resting cells tend to disappear. New meristems are formed in the tissue and these give rise to undifferentiated parenchymatous cells without any of the structural order that was characteristic of the organ or tissue from which they were derived. Although callus remains unorganised, as growth proceeds, some kinds of specialised cells may again be formed. Such differentiation can appear to take place at random, but may be associated with centres of morphogenesis, which can give rise to organs such as roots, shoots and embryos. A new production of plants from unorganised cultures is often referred to as plant regeneration. Although most experiments have been conducted with the tissues of higher plants, callus cultures can be established from gymnosperms, ferns, mosses and thallophytes. Many parts of a whole plant may have an ultimate potential to proliferate *in vitro*, but it is frequently found that callus cultures are more easily established from some organs than others. Young meristematic tissues are most

suitable, but meristematic areas in older parts of a plant, such as the cambium, can give rise to callus. The choice of tissues from which cultures can be started is greatest in dicotyledonous species. A difference in the capacity of tissue to give rise to callus is particularly apparent in monocotyledons. In most cereals, for example, callus growth can only be obtained from organs such as zygotic embryos, germinating seeds, seed endosperm or the seedling mesocotyl, and very young leaves or leaf sheaths, but so far never from mature leaf tissue (e.g. Green and Phillips, 1975; Dunstan *et al*., 1978). In sugar cane, callus cultures can only be started from young leaves or leaf bases, not from semi-mature or mature leaf blades. Even closely associated tissues within one organ may have different potentials for callus origination. Thus when embryos of *Hordeum distichum* at an early stage of differentiation are removed from developing seeds and placed in culture, callus proliferation originates from meristematic mesocotyl cells rather than from the closely adjacent cells of the scutellum and coleorhiza (Granatek and Cockerline, 1979).

The callus formed on an original explant is called ‘primary callus’. Secondary callus cultures are initiated from pieces of tissue dissected from primary callus (. Subculture can then often be continued over many years, but the longer callus is maintained, the greater is the risk that the cells thereof will suffer genetic change. Callus tissue is not of one single kind. Strains of callus differing in appearance, colour, degree of compaction and morphogenetic potential commonly arise from a single explant. Sometimes the type of callus obtained, its degree of cellular differentiation and its capacity to regenerate new plants, depend upon the origin and age of the tissue chosen as an explant. Loosely packed or ‘friable’ callus is usually selected for initiating suspension cultures. Some of the differences between one strain

of callus tissue and another can depend on which genetic programme is functioning within the cells (epigenetic differences). Variability is more likely when callus is derived from an explant composed of more than one kind of cell. For this reason there is often merit in selecting small explants from only morphologically uniform tissue, bearing in mind that a minimum size of explant is normally required to obtain callus formation. The genetic make up of cells is very commonly altered in unorganised callus and suspension cultures.Therefore another reason for cell strains having different characteristics, is that they have become composed of populations of cells with slightly different (Yeoman and Forche, 1980).

The callus tissue is modified into organized into organized small bipolar structure which later on bears cotyledons resembling zygotic embryo. The bipolar structure of the somatic embryo contains both shoot and root meristems. As the embryo develop, they progress through the distinct structural steps of the proembryo, globular, heart, torpedo and mature stages. The various stages in somatic embryo development are usually described as follows (Giri and Giri, 2007):

Proembryo : Small clusters of meristematic cells from which somatic embryos will arise

Globular stage : Larger groups of cells not yet having a definite embryoid shape

Heart stage : A characteristic three-lobed stage where cotyledonary initials are separated from the root pole

Torpedo stage : An elongated form of the heart-shaped embryo

Mature stage : Discernible small ‘seedling’ with primary root and shoot

2.4.3 Cell suspension cultures

Unorganised plant cells can be grown as callus in aggregated tissue masses, or they can be freely dispersed in agitated liquid media. Techniques are similar to those used for the large-scale culture of bacteria. Cell or suspension cultures, as they are called, are usually started by placing an inoculum of friable callus in a liquid medium. Under agitation, single cells break off and, by division, form individual cells and other small cell groups. It is not always necessary to have a previous callus phase before initiating suspension cultures. For example, leaf sections of Chenopodium rubrum floated on Murashige and Skoog (1962) medium in the light, show rapid growth and cell division in the mesophyll, and after 4 days on a rotary shaker they can be disintegrated completely to release a great number of cells into suspension (Geile and Wagner, 1980). Because the walls of plant cells have a natural tendency to adhere, it is not possible to obtain suspensions that consist only of dispersed single cells. Some progress has been made in selecting cell lines with increased cell separation, but cultures of completely isolated cells have yet to be obtained. The proportion and size of small cell aggregates varies according to plant variety and the medium in which the culture is grown. As cells tend to divide more frequently in aggregates than in isolation, the size of cell clusters increases during the phase of rapid cell division. Because agitation causes single cells, and small groups of cells, to be detached, the size of cell clusters decreases in batch cultures as they approach a stationary growth (Geile and Wagner, 1980). The degree of cell dispersion in suspension cultures is particularly influenced by the concentration of growth regulators in the culture medium. Auxinic growth regulators increase the specific activity of enzymes, which bring about the dissolution of the middle lamella of plant cell walls (Torrey and

Reinert, 1961). Thus by using a relatively high concentration of an auxin and a low concentration of a cytokinin growth regulator in the culture medium, it is usually possible to increase cell dispersion (Narayanaswamy, 1977). However, the use of high auxin levels to obtain maximum cell dispersion will ensure that the cultured cells remain undifferentiated. This may be a disadvantage if a suspension is being used to produce secondary metabolites. Well-dispersed suspension cultures consist of thin-walled undifferentiated cells, but these are never uniform in size and shape. Cells with more differentiated structure, possessing, for example, thicker walls and even tracheid-like elements, usually only occur in large cell aggregates (Narayanaswamy, 1977).

The use of suspension cultures in plant propagation :

The growth of plant cells is more rapidin suspension than in callus culture and is also more readily controlled because the culture medium can be easily amended or changed. Organs can be induced to develop in cell suspensions: root and shoot initiation usually commences in cell aggregates. Somatic embryos may arise from single cells. Cells from suspensions can also be plated onto solid media where single cells and/or cell aggregates grow into callus colonies from which plants can often be regenerated. For these reasons suspension cultures might be expected to provide a means of very rapid plant multiplication. There are two methods:

• plants may be obtained from somatic embryos formed in suspensions. Once embryos have been produced, they are normally grown into plantlets on solid media.

• cells from suspensions are plated onto solid media where single cells and/or cell aggregates grow into callus colonies from which plants can often be regenerated.

It is relatively easy to induce callus and suspension cultures from many species, including woody species. So far, There are reported have been able to induce organogenesis and/or embryogenesis with very few species. Polyploid cells and cells with various types of chromosomal variations from the normal for the species have often been reported from callus and suspension cultures and thus plants which are induced to form from such cultures, either by the induction of adventitious buds or by embryogenesis may rise from cells of an abnormal type. Nevertheless, the presence of cells with other than normal genomes does not necessarily prevent the formation of adventitious buds and embryos with the normal chromosomal type of the species. On the other hand, this variation in genome could lead to the formation of interesting and valuable new strains. However, clonal propagation can use callus and suspension culture methods with extreme caution. But for somaclonal variation it might be of considerable importance in plant breeding. The major requirements for using somaclonal variation in plant breeding are :

- Techniques to culture tissues and regenerate genetically stable plants
- Techniques to screen regenerated plants for desired variants

2.4.4 Embryogenesis in suspension cultures :

Suspension cultures can sometimes be initiated from embryogenic callus tissue, and the cells still retain the capacity to regenerate somatic embryos freely. Obtaining such cultures is not always a simple matter, for the auxin levels that are often used to promote cell dispersion may result in the loss of morphogenic capability.

Embryogenic cell suspensions are most commonly initiated from embryogenic callus that is placed in liquid medium on a shaker. Embryoids were induced to develop into somatic seedlings when plated onto an agar medium without growth regulators, or with lower levels of auxin than used at the previous stage.

2.4.4.1 Subcultering :

Once a particular kind of organised or unorganised growth has been started *in vitro*, it will usually continue if callus cultures, suspension cultures, or cultures of indeterminate organs are divided to provide new explants for culture initiation on fresh medium. Subculturing often becomes imperative when the density of cells, tissue or organs becomes excessive; to increase the volume of a culture; or to increase the number of organs (e.g. shoots or somatic embryos) for micropropagation. The period from the initiation of a culture or a subculture to the time of its transfer is sometimes called a passage. The first passage is that in which the original explant or inoculum is introduced. Suspensions regularly subcultured at the end of the period of exponential growth can often be propagated over many passages. However, many cultures reach a peak of cell aggregation at this time and aggregation often becomes progressively more pronounced in subsequent passages (Street, 1977).

Subculture is therefore more conveniently carried out during the stationary phase when cell aggregation is least pronounced. Rapid rates of plant propagation depend on the ability to subculture shoots from proliferating shoot or node cultures, from cultures giving direct shoot regeneration, or callus or suspensions capable of reliable shoot or embryo regeneration. A further reason for transfer, or subculture, is that the growth of plant material in a closed vessel eventually leads to the

accumulation of toxic metabolites and the exhaustion of the medium, or to its drying out. Thus, even to maintain the culture, all or part of it must be transferred onto fresh medium. Callus subcultures are usually initiated by moving a fragment of the initial callus (an inoculum) to fresh medium in another vessel. Shoot cultures are subcultured by segmenting individual shoots or shoot clusters. The interval between subcultures depends on the rate at which a culture has grown: at 25°C, subculturing is typically required every 4-6 weeks. In the early stages of callus growth, it may be convenient to transfer the whole piece of tissue to fresh medium, but a more established culture will need to be divided and only small selected portions used as inocula. Regrowth depends on the transfer of healthy tissues. Decontamination procedures are theoretically no longer necessary during subculturing, although sterile transfer procedures must still be used (Street, 1977). However, when using shoot or node cultures for micropropagation, some laboratories do re-sterilise plant material at this stage as a precaution against the spread of contaminants. Cultures which are obviously infected with micro-organisms should not be used for subculturing and should be autoclaved before disposal.

2.4.4.2 Subcultering hazards :

There are several hazards in subculturing which are discussed more fully in other chapters of this dissertation. Several kinds of callus may arise from the initial explant, each with different morphogenic potential. Strains of callus tissue capable of giving rise to somatic embryos and others without this capability can, for instance, arise simultaneously from the culture of grass and cereal seed embryos. Careful selection of the correct strain is therefore necessary if cultures capable of producing

somatic embryos are ultimately required. Timing of the transfer may also be important, because if left alone for some while, non-embryogenic callus may grow from the original explant at the expense of the competent tissue, which will then be obscured or lost. Although subculturing can often be continued over many months without adverse effects becoming apparent, cultures of most unorganised cells and of some organised structures can accumulate cells that are genetically changed. This may cause the

characteristics of the culture to be altered and may mean that some of the plants regenerated from the culture will not be the same as the parent plant. Cultures may also inexplicably decline in vigour after a number of passages, so that further subculture becomes impossible (Street, 1977).

2.5 The components of plant tissue culture media :

2.5.1 Macro- and Micro-nutrients :

- Inorganic medium components :

Plant tissues and organs are grown *in vitro* on artificial media, which supply the nutrients necessary for growth. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium used. For healthy and vigorous growth, intact plants need to take up from the medium:

• relatively large amounts of some inorganic elements (the so-called major plant nutrients): ions of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and sulphur (S)

• small quantities of other elements (minor plant nutrients or trace elements): iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo). According to Epstein (1971), an element can be considered to be essential for plant growth if:

1. a plant fails to complete its life cycle without it
2. its action is specific and cannot be replaced completely by any other element
3. its effect on the organism is direct, not indirect on the environment
4. it is a constituent of a molecule that is known to be essential

Plant tissue culture media provide not only these inorganic nutrients, but usually a carbohydrate (sucrose is most common) to replace the carbon which the plant normally fixes from the atmosphere by photosynthesis. To improve growth, many media also include trace amounts of certain organic compounds, notably vitamins, and plant growth regulators.

- Organic supplements :

Growth and morphogenesis of plant tissue cultures can be improved by small amounts of some organic nutrients. These are mainly vitamins (including some substances that are not strictly animal vitamins), amino acids and certain undefined supplements. The amount of these substances required for successful culture varies with the species and genotype, and is probably a reflection of the synthetic capacity of the explant.

2.5.2 Growth regulators :

Some chemicals occurring naturally within plant tissues (*i.e.* endogenously), have a regulatory, rather than a nutritional role in growth and development. These compounds, which are generally active at very low concentrations, are known as plant hormones (or plant growth substances). Synthetic chemicals with similar physiological activities to plant growth substances, or compounds having an ability to modify plant growth by some other means, for example polyamines, are usually termed plant growth regulators. Some of the natural growth substances are prepared synthetically or through fermentation processes and can be purchased from chemical suppliers. When these chemicals have been added to plant tissue culture media, they are termed plant growth regulators in this dissertation, to indicate the fact that they have been applied from outside the tissues (i.e. exogenously). There are several recognised classes of plant growth substance. Until relatively recently only five groups were recognised namely:

- auxins
- cytokinins
- gibberellins
- ethylene
- abscisic acid

Auxins and cytokinins are by far the most important for regulating growth and morphogenesis in plant tissue and organ cultures; in these classes, synthetic regulators have been discovered with a biological activity, which equals or exceeds that of the equivalent natural growth substances. No chemical alternatives to the natural gibberellins or abscisic acid are available, but some natural gibberellins are extracted

from cultured fungi and are available for use as exogenous regulants. However, several classes of chemicals, which are highly effective in blocking the synthesis of gibberellins within the plant, are very effective growth regulators. They are usually termed antigibberellins (or growth retardants). These however, can also affect the synthesis of other classes of hormone or growth regulator such as abscisic acid, sterols or brassinosteroids.

2.6 Genetic stability :

In some crop plants, the genetic differences between plants derived from callus and suspension cultures are considerable, and are sufficient to have attracted the interest of plant breeders as a new source of selectable variability. However, plants obtained from callus lines with a high degree of morphogenic competence, appear to be much more uniform genetically. Care must be taken though to see that primary explants are not taken from plant tissue likely to be endopolyploid. Subsequent exposure to high levels of growth substances such as 2,4-D should also be avoided as far as possible. Genetic stability of plants from highly competent callus cultures may be assisted by the continual presence of superficial meristems. These probably repress shoot formation from cells within the callus mass (Hussey, 1983).

2.7 Doubling chromosome number :

Doubling the chromosome numbers of pollen-derived plant is difficult. Colchicine, the chemical most commonly used as a doubling agent, is not successful in all cases. Because colchicines acts on mitosis, the apex of young haploid plant or the pollen itself at the time of the first pollen mitosis are good parts to treat. A method

that gives a good percentage of doubling in barley is to submerge the apical part of the haploid plant still growing in test tube in a solution of colchicines (Jensen, 1974). In pollen-derived plants, the highest percentage of diploid plants was produced when the anther was treated by colchicines at the time of the first pollen mitosis (Nitsch, 1977). Doubling the chromosome number at the unicellular level has the advantage of uniformity. There will be no somaclonal or genetic variation in the plant and the diploid tissue grows better than the haploid (Jensen, 1974). In order to avoid genetic chimera and to obtain a high regeneration rate of diploidized plants in anther culture, it is considered to be important to induce chromosome doubling at the first pollen division. In the present some research could obtain a high proportion of fertile regenerants by employing the simple and efficient chromosome doubling method in which rice spikes were treated with colchicine solution before anther culture.

2.8 Somaclonal and gametoclonal variations :

Somaclonal variation refers to the heritable changes that result from *in vitro* procedures (Skirvin, 1978). A small percentage of plants regenerated adventitiously from tissue culture exhibit traits not previously observed. When the variation is heritable it is said to be somaclonal variation. Somaclonal variation has been implicated in changes in characters such as growth habit floral expression, fruit quality, desease and pesticide resistance, the presence or absence of thorns and flower colour. It often occurs at higher frequencies than chemical or radiation induced mutation, making it a viable alternative to mutagenesis.

Scientists interested in inducing somaclonal variation propagate their plants rapidly *in vitro* on medium supplemented with growth regulators that are expected to

stimulate callus and adventitious buds. The amount of variability that can be expected will vary with the clone. If this rate of variability is too low or not reliable, it may be increased by repeated transfer, the use of mutagenic agents, or by applying selection pressure to single cells for stress conditions, ability to resist toxins, or to utilize specific metabolites. The exploitation of somaclonal variation seems especially applicable to older vegetatively propagated cultivars or clones which could be expected to have accumulated large numbers of mutant somatic cells after decades or even centuries of vegetative propagation (Skirvin, 1978).

2.8.1 Sources of somaclonal variation :

1. Pre-existin variation: Cell division is a precisely controlled event that normally yields identical copies of the parental cell. If the control mechanism falters, replicate errors can result in cellular abnormalities.

2. Tissue culture induced variation: When plant explants are grown *in vitro*, the tissue culture environment itself appears to upset normal controls of cell division and chromosome distribution to result in aberrant cells.

2.8.2 Factors determining somaclonal variation :

1. Type of growth regulator: Growth regulators like 2,4-D, 2,4,5-T and BAP enhance the rapid proliferation of disorganized cell. High concentration leads to alteration in the ploidy levels.

2. Cultivars: The amount of variation arise through *in vitro* is not same for all the cultivars of a species.

3. Ploidy level: High somaclonal variation has direct correlation with polyploidy or high chromosome level.

4. Length of time *in vitro*: One of the important factors. It is must to limit the number of subcultures that can made from an explants. Also if we maintain the cultures, established from a particular explants more than a year, it will lead to somaclonal variation.

5. Proliferation rate: Normally excessive proliferation will lead to more variation. In case of pineapple by conventional cuttings we can produce 4-5 plants in a year but in case of *in vitro* propagation 30-50 plants per month can be obtained. Here the variation will be more than 50% which is unacceptable commercially.

The cause of somaclonal or genetic variation during tissue culture might be either the explant variation or variation during the process. Genetic variation is beneficial in breeding as it brings about various new variations even though there seems to be unprofound understanding about genetic mechanism at present. This method can be applied in many breeding such as wheat, rice, corn, barley, potato and sugar cane.

2.8.3 Applications somaclonal variation in plant breeding :

Somaclonal variation and gametoclonal variation are the important sources of introducing genetic variations that could be of value to plant breeders. Single gene mutations in the nuclear or organelle genome usually provides the best available variety *in vitro*, which has a specific improved character. Somaclonal variations are

used to uncover new variants retaining all the favourable characters along with an additional useful trait. For example, Resistance to disease or a herbicide. These variants can then be field tested to ascertain their genetic stability. Gametoclonal variation is induced by meiotic recombination during the sexual cycle of the F1 hybrid results in transgressive segregation to uncover unique gene combinations. Though diploidization of callus cells and haploid regenerants have been examined, it is difficult because of the low frequency in diploidization and the high rate of chromosomal chimera (Shahjahan *et al.*, 1992).

2.9 Chromosomal analysis of *in vitro* cultured tissue and regenerated plants :

There are always possibilities of changes in the genetic setup of a plant cell, protoplasts and tissue when subjected to *in vitro* culture. The manifestation of the changes in chromosome structure and behavior may be diverse. They include most frequent variations like chromosome number, rearrangements of the chromosomes, chromosome breakage, chromosome translocations, even deletions of chromosome parts and phenomenon like mitotic crossing over. If one want to evaluate these chromosomal variations of *in vitro* cultured tissue and regenerated plants, similar traditional methods and protocols in cytological research are followed. Besides other chromosomal variations, most importantly the chromosome number analysis, is an essential aspect of cytological study of *in vitro* cultured cell and tissue. Different aspects of plant tissue culture such as cell suspension, protoplast, callus culture, multiple shoot culture, anther culture, organogenesis, embryogenesis and production of somatic hybrids through protoplast fusion needs chromosome analysis for better

planning of the experiments. The metaphase chromosome analysis in mitosis and their behavior in meiosis generally studied may give preliminary information about genetic variation pertaining to chromosome number (Giri and Giri, 2007).

The chromosomal studies in turn may help to categorize cultured tissues and regenerated plants, and can modify protocols for tissue culture and regeneration methods according to the objectives of the research and select suitable regenerated plants for further experimentation and evaluation.

2.10 Auxin effect in tissue culture :

In tissue culture, depending on other hormones present in the medium, changes in auxin concentrations may change the type of growth, *e.g*., stimulation of root formation may switch to callus induction etc. In this respect, each tissue culture system is unique, and the effects of different concentrations of auxins and other hormones must be tested for each case individually and only to some extent can the results can be transferred to other cultures.

2.10.1 Induction of callus growth :

An auxin is generally required for the induction of callus from explants. Applied auxins seem to be capable of fundamentally altering the genetically programmed physiology of whole plant tissues, which had previously determined their differentiated state. Cells, which respond to auxin, revert to a dedifferentiated state and begin to divide. How auxin brings about this reprogramming is understood only to a very limited extent. Lo Schiavo *et al.,* (1989) found that auxins cause DNA to become more methylated than usual and suggested that this might be necessary for

the reprogramming of differentiated cells. Thus, tissue-specific programmes specifically associated with differentiation would be eradicated by hypermethylation, with perhaps a small fraction of the cells reaching an ultimate state of dedifferentiation in which they become capable of morphogenesis, or embryogenesis (Terzi and Lo Schiavo, 1990). A high rate of DNA methylation was found in the early somatic embryo stage in cultures of *Cucurbita pepo* L (Leljak-Levanic *et al*., 2004).

Auxins promote cell dispersion in suspension cultures while cytokinins tend to cause cell aggregation. The relatively high levels of auxin added to liquid media to obtain dispersion will prevent morphogenesis, but might induce embryogenesis if the cells are still competent. Whereas cytokinins tend to promote the formation of chlorophyll in callus and suspension cultures, auxins can be inhibitory.

2.10.2 Organ cultures :

An auxin is almost invariably required to promote the initial growth of meristem and shoot tip explants. A low concentration of auxin is often beneficial in conjunction with high levels of cytokinin at Stage II when shoot multiplication is required, although in some cases cytokinin alone is sufficient. It is important to choose an auxin at a concentration that will promote growth without inducing callus formation. The induction of rhizogenesis usually requires an adjustment in the levels of auxins and cytokinins. Rhizogenesis is usually achieved by treatment with auxin alone. Also, development of lateral roots is stimulated by auxin as was demonstrated in *Panax ginseng*, where IBA was shown to be more effective than NAA (Kim *et al.*, 2003). Exogenous cytokinins are commonly inhibitory (Reid and Howell, 1995). Auxin-induced root formation is thought to require, or induce, the promotion of

polyamine synthesis (Friedman *et al*., 1985). Sometimes tissues, organs or strains of cells arise that are able to grow without the addition of any auxin to the medium.

2.10.3 Embryogenesis :

The process of somatic embryogenesis is often initiated in media containing high levels of auxins (especially 2,4-D), but embryos usually do not develop further until the auxin concentration is reduced. Sharp *et al.*, (1980) proposed that auxin induces an embryogenic determination in a proportion of the cells in callus or suspension cultures but at the same time causes these induced cells to cease further development into embryos. It was suggested that division of the pro-embryogenic cells and their development into embryos is only resumed at lower auxin concentrations. There are, however, many recorded exceptions to this general observation, where somatic embryos are induced even in cultures grown on media devoid of auxins. It is possible that in these instances, embryogenesis has been induced by endogenous auxin, the concentration of which has then been subsequently reduced by metabolism to permit embryo formation. In alfalfa, low 2, 4-D concentration gives rise to callus from leaf explants, while higher 2,4-D levels induce formation of embryo-like structures (Fehér *et al*., 2002). Withdrawal of auxin from the inducing medium is associated with cell death and extracellular acidification in cultures of *Norway spruce* (Bozhkov *et al*., 2002). The discovery that embryo formation in carrot can be regulated by pH may imply that at least some of the regulatory effects of auxins on the formation and maintenance of embryogenic cultures can be ascribed to their capacity to reduce intracellular pH. Embryo formation coincides with the withdrawal of auxin and a rise in cellular pH. Formation

of cellular pH gradients may be important in the formation of embryogenic cells in alfalfa (Pasternak *et al*., 2002). Thus it cannot be assumed that pH is the only controlling factor: a common physiological mechanism by which such different stimuli can induce embryogenesis has yet to be demonstrated. In the induction of somatic embryogenesis from immature cotyledons of *Glycine max*, Lazzeri *et al*., (1988) discovered a highly significant interaction between the concentration of auxin and sucrose in the medium. The number of embryos obtained was reduced if the concentration ratio of auxin: sucrose was high, or *vice versa*.

2.11 Growth regulator shock :

Among the external factors the exogenously applied hormones, mainly auxins such as 2,4-D, play a critical role in the reactivation of the cell cycle and the initiation of the embryo formation. Application of high concentrations of 2,4-D in the culture medium itself is a stress signal since embryogenic induction requires the use of a physiological auxin concentrations that inhibit the callus growth. The inductive effect of a short auxin shock can be clearly demonstrated with help of microcallus suspentions from alfalfa (*Medicago sativa*). Treatment of dedifferentiated cells grown in the presence of weak auxin: NAA with 100 µM 2,4-D for a few minutes up to a few hours is sufficient to induce embryo formation of embryogenic somatic cells. In addition, the use of these culture allows the exact timing of the inductive phase. In contrast, the proembryogenic nature of carrot suspension cultures make it difficult to determine the time of commitment of somatic cell towords embryogenesis. Differences between carrot and alfalfa embryogenic culture systems are summarized by Dudits *et al.* (1991).

2.12 Synthetic seeds (Artificial seeds) :

Another essential of tissue culture was to give more survival rate opportunity of embryoid planted *in vivo* condition. Generally, plantlets produced *in vitro* would be adjusted in order to enable them to germinate in that condition, most of them were not only less in germination rate, but also took long period of time in adjusting before planting. The method in strengthening embryos by producing synthetic seed coat for embryos, known as “Synthetic seed or artificial seed production technique”, before planting *in vivo* was later found.

The synthetic seed technology has been developed to use somatic embryos and/or other micropropagules as seed analogues successfully in the field or greenhouse, and their mechanical planting at a commercial level. The technology provides methods for preparation of seed analogues called synthetic seeds or artificial seeds from the micropropagules like somatic embryos, axillary shoot buds, apical shoot tips, embryogenic calli as well as protocorm or protocormlike bodies. For the last fifteen years, intensive research efforts have been made on synthetic seed production in a number of plant species. Despite these researches, practical implementation of the technology is yet to be fully realized due to limitations encountered with the production, development, maturation and subsequent conversion of the micropropagules into plantlets under *in vitro* or *ex vitro* conditions. The present article focuses on the technology developed, its achievements and prospects as well as limitations resisting the application of the synthetic seed technology.

Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions, and

that retain this potential also after storage (Capuano *et al*., 1998). Earlier, synthetic seeds were referred only to the somatic embryos that were of economic use in crop production and plant delivery to the field or greenhouse (Gray and Purohit, 1991; Janick *et al*., 1993. In the recent past, however, other micropropagules like shoot buds, shoot tips, organogenic or embryogenic calli, etc. have also been employed in the production of synthetic seeds. Thus, the concept of synthetic seeds has been set free from its bonds to somatic embryogenesis, and links the term not only to its use (storage and sowing) and product (plantlet) but also to other techniques of micropropagation like organogenesis and enhanced axillary bud proliferation system. Implementation of synthetic seed technology requires manipulation of *in vitro* culture systems for large-scale production of viable materials, that are able to convert into plants, for encapsulation. Somatic embryogenesis, organogenesis and enhanced axillary bud proliferation systems are the efficient techniques for rapid and largescale *in vitro* multiplication of elite and desirable plant species. Through these systems a large number of somatic embryos or shoot buds are produced which are used as efficient planting material as they are potent structures for plant regeneration either after having minor treatment or without any treatment with growth regulator(s). Because the naked micropropagules are sensitive to desiccation and/or pathogens when exposed to natural environment, it is envisaged that for largescale mechanical planting and to improve the success of plant (*in vitro* derived) delivery to the field or greenhouse, the somatic embryos or even the other micropropagules useful in synthetic seed production would necessarily require some protective coatings. Encapsulation is expected to be the best method to provide protection and to convert the *in vitro* derived propagules into ‘synthetic seeds’ or ‘synseeds’ or ‘artificial seeds’

(Redenbaugh, 1993). The encapsulation technology has been applied to produce synthetic seeds of a number of plant species belonging to angiosperms and gymnosperms. Nevertheless, their number is quite small in comparison to the total number of plant species in which *in vitro* regeneration system has been established. Production of artificial seeds has unravelled new vistas in plant biotechnology. The synthetic seed technology is designed to combine the advantages of clonal propagation with those of seed propagation and storage. Despite the fact that the technology is an exciting and rapidly growing area of research in plant cell and tissue culture, there are many limitations for its practical use.

Techniques of micropropagation or *in vitro* cultivation have emerged as alternatives for species that do not have the property of producing viable seeds, that is, species that cannot germinate and develop adequately in their natural environment (González *et al*., 2004). Among the micropropagation techniques is somatic embryogenesis or the regeneration of embryos based on the vegetative tissue of *N. alpina* as an efficient technique that allows for the mass propagation of selected genotypes, for productive and conservation purposes (Castellanos *et al*., 2004). The success of this technique depends on the development of a series of processes that influence the genotype of the mother or donating explant and the concentration of exogenous growth regulators, which in adequate combinations would allow for obtaining a determinant embryogenic response for the production of somatic embryos (SE) (Guerra *et al.*, 2001). The SE can be immersed in a protective matrix constituting an artificial or synthetic seed, providing a convenient method for the propagation by cloning of elite plant varieties or species that are difficult to propagate in their natural environment (Fuji *et al*., 1987). The first indications of artificial seed propagation

were reported in annual crops such as alfalfa (*Medicago sativa* L.) and sugar cane (*Saccharum* spp.). Currently, systems of artificial seed production have progressed substantially in this area, the most advanced being in seeding under *ex vitro* or field conditions, obtaining high percentages of conversion to plants (Fuji *et al*., 1987; Nieves *et al*., 2003). However, the germination rates and the subsequent conversion to plants of somatic embryo of different woody species are still low, owing mainly to deficient and asynchronic maturation of the embryonic pole, which makes the terminal stages of the process difficult (Tapia *et al*., 1999; Castellanos *et al.*, 2004). Several researchers suggest that to control growth and facilitate the germination of SE, the synthetic endosperm can simulate an endosperm of sexual origin, containing one or several compounds such as: nutrients, growth regulators, anti-pathogens, herbicides, bio-controllers and bio-fertilizers, among others, with the aim of ensuring the conversion of the plant and its development in the field (Castillo *et al*., 1998; Kumar *et al*., 2004; Malabadi and Van Staden, 2005). The composition of the protective matrix should allow for the growth of the encapsulated embryo, providing mechanical resistance according to the available energy of the embryo, given that an excessively hard endosperm results in energy loss and weak or nil growth of the encapsulated ES (Jiménez and Quiala, 1998; González *et al*., 2004). The literature notes the use of different substances (agar, gelrite) to encapsulate embryos, with the manipulation of the concentration of sodium alginate and the exposure time to the complexing agent calcium chloride reporting the best results of germination and conversion to plants in woody species (Patel *et al*., 2000; Maruyama *et al*., 2003; Utomo *et al*., 2008).

According to the new conception “artificial seed” comprises a capsule prepared by coating a culture matter, like a tissue piece or an organ which can grow into a complete plant body, along with nutrients, with an artificial film/covering. The ability of the technology to handle a small (few mm) propagule/somatic embryo rather than a large plantlet provides tremendous flexibility for plant propagation. Planting, storage and shipping becomes easy with efficient transfer of technology from lab to land (Giri and Giri, 2007).

Synthetic seeds or artificial seeds depend on plant asexual propagation with tissue culture in somatic cell through somatic embryogenesis. This process is developed to somatic embryos or embryoids without natural fertilization of sex cells. In synthetic seed production, somatic embryos are encapsulated with gelatinous solution: alginate or other substances. These substances not only function as synthetic seed coats, that is, they protect embryos from any dangers like those in real seeds, but they are also the synthetic food storages (Redenbaugh *et al.*, 1987; Gray, 1987). Additionally, other explants which develop to growing plantlet such as shoot, root, bud, anther and callus can be encapsulated with gelatinous solution in order to resemble real seeds.

2.12.1 Advantages of synthetic seeds :

- Helps in clonal propagation to replace traditional seed propagation. A replacement for hand pollinated hybrid plant
- Carriers for beneficial microorganisms, pesticides and growth regulators
- Not season dependent
- Role of different embryonic constituents can be studied

2.12.2 Limitations of synthetic seeds :

- Limited production of viable micropropagules useful in synthetic seed production
- Anomalous and asynchronous development of somatic embryos
- Improper maturation of the somatic embryos that makes them inefficient for germination and conversion into normal plants
- Lack of dormancy and stress tolerance in somatic embryos that limit the storage of synthetic seeds
- Poor conversion of even apparently normally matured somatic embryos and other Micropropagules into plantlets that limit the value of the synthetic seeds and ultimately the technology itself

Development of artificial seeds requires sufficient control of somatic embryogeny from the explants to embryo production, embryo development and their maturation as well. The mature somatic embryos must be capable of germinating out of the capsule or coating to form vigorous normal plants. A number of researchers have tried to improve the quality (Attree *et al*., 1995) and quantity (Burns and Wetzstein, 1997) of somatic embryos via modification of culture conditions, such as, medium composition, growth regulators (types and concentrations), physical state of the medium, as well as incubation conditions like temperature, illumination, etc.

Synthetic seeds were appropriate for many economic plants such as decorative plant, vegetable, horticulture and hybrid cereal (Gray, 1987). The reasons to use synthetic seeds in these plants were: they could be produced in high quantity at a time and in all seasons; the production requires short period of time and used less labours and planting areas; plants regenerated from synthetic seeds would receive identical

parent characteristics because of its asexual propagation; synthetic seeds' quality would be stable and pathogen–free before applying in efficiency germplasm collection (Redenbaugh *et al*., 1991). However, success of the synthetic seed technology is constrained due to scarcity and undesirable qualities of somatic embryos making it difficult for their development into plants. The choice of coating material for making synseeds is also an important aspect for synseed production.

Based on technology established so far, two types of synthetic seeds are known: *desiccated* and *hydrated*. The desiccated synthetic seeds are produced from somatic embryos either naked or encapsulated in polyoxyethylene glycol (Polyoxr) followed by their desiccation. Desiccation can be achieved either slowly over a period of one or two weeks sequentially using chambers of decreasing relative humidity, or rapidly by unsealing the petri dishes and leaving them on the bench overnight to dry. Such types of synseeds are produced only in plant species whose somatic embryos are desiccationtolerant. On the contrary, hydrated synthetic seeds are produced in those plant species where the somatic embryos are recalcitrant and sensitive to desiccation. Hydrated synthetic seeds are produced by encapsulating the somatic embryos in hydrogel capsules.

The capsules' strength depended on the ratio between guluronic acid per mannuronic acid, cation and period of time in complexation. However, algenic acid concentration had an effect on germination rate because of the capsules' strength. Fourré *et al*. (1997) had found that 4 % weight by volume alginate would inhibit somatic embryo germination while 1, 2 and 3 % weight by volume alginate could promote high germination rate. However, 1 % weight by volume alginate tended to produce very weak capsules which were difficult for the transportation. The capsule

size could be controlled by sodium alginate viscosity and the diameter at the end of the dropper (Redenbaugh *et al*., 1988).

Dried synthetic seeds enabled embryos to had dormancy similar to orthodox (Gray, 1987; Kitto and Janick., 1985). Water evaporation dehydration until the seeds reached 90 % water loss could prolong storage and was very adventagous in germplasm collection. Senaratna *et al*. (1990) had discovered found that dehydrated alfalfa somatic embryos would strengthen plants better than non-dehydrated ones did.

The seed laboratory, department of Agronomy, Chiang Mai University had succeeded in developing tissue culture and synthetic seed production techniques in monocotyl and dicotyl plants which are economically important. Thobunluepop (2003) had studied the enhancement of sweet corn synthetic seed viability under various storage condition by comparing callus growth in MS media with that in N_6 media which then discovered that N_6 media which added 60 g/l sucrose and 3 mg/l 2,4-D could induce and increase callus. Moreover, adding benomyl during synthetic seed production would increase 41 % germination, higher than the study of Green and Phillips (1975) which had 10-15 % germination. Similarly, Boonpeng (2003) had produced sugarcane dried synthetic seed by treating MS media which added 0.1 mg/l ABA and found that it could induce desiccation tolerance in synthetic seeds and prevent precocious germination at 4±2 °C, as well. The 60 % germination, which was higher than the study of Aflab *et al*. (1996), would occur when planting the seeds in MS media. In vegetable plant, Preamvaranon (2005) had studied the viability enhancement of sweet pepper synthetic seeds by treating MS media in 0.5 mg/l ABA with synthetic seed dehydration until it reached 80 % water loss and then stored at 25±2 °C. The seeds, as from the research results, could be stored for 2 weeks without

precocious germination during the storage. After 3 days of planting in MS media, stored synthetic seeds provided 86 % germination with 97 % normal seedlings, higher than the study of Buyukalaca and Mavituna (1996) which had 80 % germination. Furthermore, Improving survival of pollen in *in vitro* culture increases the chances of inducing androgenesis. The cold shock given flowers before planting the anther to induce the pollen toward androgenesis also affects the viability of the pollen in culture.