CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Growth is an irreversible increase in the plant sizes. Like other organisms, plants are made up of cells and growth involves an increase in cell numbers through cell division and an increase in cell size. Cell division itself is not growth as each new cell is exactly half the size of the cell from which it was formed. As each cell has a maximum size, cell division is considered as providing the potential for growth.

While growth in plants consists of an increase in both cell number and cell size, animal growth is almost wholly the result of an increase in cell numbers. Another important difference in growth between plants and animals is that animals are determinate in growth and reach a final size before they are mature and start to reproduce. Plants have indeterminate growth and, as long as they live, the addition of new organs and tissues is continuous. In a plant, new cells are produced all the time, and some parts such as leaves and flowers may die, while the main body of the plant persists and continues to grow. The basic processes of cell division are similar in plants and animals, though the presence of a cell wall and vacuole in plant cells means that there are certain important differences. This is particularly true in plant cell enlargement, as plant cells, being restrained in size by a cellulose cell wall, cannot grow without an increase in the wall. Plant cell growth is thus largely a property of the cell wall.

Wareing and Phillips (1978) defined plant growth as a quantitative changes that occur during development stage of a plant which involves the increase of cells through cell division and also the increase of cell size. According to Simpkins and William (1982), growth is a continuous process until it reaches a level in which throughout the development process, growth and development occur at the same time for all living organisms. Growth takes place when there is a response to the environment (Raven and Johnson, 2001). As other living organisms, plants developed through the genetic information that they carry. Nevertheless, the genetic information is influenced by the environment.

According to Esau (1977), growth is a process that involves the establishment of new cells along with the continuous increase and development of the cells. The increase of cells is the result of cell division and the increase of cell size. The division of vegetative cells through mitosis is an important process in plant development. A mature plant composed of a number of cells which are different in types and sizes. Physiologically, each cell has its own function and the development of various types of cells is caused by cell division. Teo (1992) defined that naturally, through development process, plant cells are capable to form a complete plant. In plant, this concept is called "totipotency of cells".

Plant cell, tissue and organ culture technique have also made an important contribution to fundamental knowledge of the cell. The history of plant tissue culture started in 1838 when Schleiden and Schwann established the 'Theory of Cells'. This

theory is the basis for totipotency concept. According to this theory, every single cell is autonomic and thus, it is capable to develop into complete individual. This theory has become the foundation of plant tissue and cell culture. The first attempt of plant tissue culture was done by Haberlandt in 1901 but failed. Haberlandt was the first to introduce plant tissue culture to the world. It has been more than a century since Haberlandt attempted the first plant tissue culture experiment.

Plant cell, tissue and organ culture has only started rapidly during the last few decades. The first successful culture was done by White (1934) when he first cultured the root and meristem of *Lycopersicon esculentum*. In the same year, Gautheret and Nobecourt initiated the first callus culture of *Daucus carota*. The development of plant tissue and organ culture in the 1950's has fully converted this field from an area of only academic interest into a major tool for the horticulture and floriculture industries through micropropagation of desired plants. The first plant growth regulators (hormones) found was indole acetic acid (IAA), a type of auxin and this created a tremendous opportunities for the *in vitro* cultures of plant tissues. Later, in 1955, kinetin (a type of cytokinin) was discovered and further stimulated plant cell and tissue culture. This rapid change has widened to agriculture industries in which important crops has successfully been improved.

Plants can be propagated in two ways, sexually and vegetatively (through asexual breeding). Both types of propagation may be impossible under certain conditions. *In vivo* vegetative propagation has played an important role in agriculture and plant breeding.

The classical methods of *in vivo* propagation often face many problems such as the propagation is too slow, difficult and sometimes expensive. Thus, *in vitro* propagation is the alternative method. Plants can be cloned disregard of the temperature and climate. It has now become possible to clone plant species through *in vitro* culture techniques that are almost impossible to clone *in vivo*, or need longer time.

In vitro propagation is also commonly known as plant tissue culture. Plant tissue culture is an important technique in producing new plants (Chu and Kurtz, 1990). Plants with valuable traits can be mass propagated through tissue culture cloning. In this application, hundreds of genetically identical plants (true-to-type) can be produced by vegetative or asexual propagation from small pieces of tissues (Bhojwani and Radzan, 1983). This has been extensively used in the flower industry where genetically identical plants can be produced from a superior parent plant. Propagation of plant tissue in sterile environment also aided the production of disease-free plants.

Generally, plant tissue culture refers to the growth and development of cells, tissues and organs which has been isolated from the mother plant and cultured *in vitro* on culture media under aseptic condition (George, 1993). Plant tissue culture has now become a powerful technique in many fields especially in biotechnology and agriculture.

In plant tissue culture, to form a complete plant, totipotency of cells involves differentiation process. Unorganized and undifferentiated tissues that are formed from the

differentiation of cells formed callus (Dodds and Roberts, 1982). Later, these tissues are able to form new organs which were not formed in the beginning and this process is called organogenesis. Generally, to form a complete plant, differentiation of cells will begin from the differentiation of shoots or roots. The formation of the structures can be done *de novo* or adventitious. The capacity of cultured plant tissues and cells to undergo morphogenesis, resulting in the formation of discrete organs or whole plants, has provided opportunities for numerous applications of *in vitro* plant biology in studies of basic botany, biochemistry, propagation, breeding and development of transgenic crop. While the fundamental techniques to achieve *in vitro* plant morphogenesis have been established for a number of years, innovations in particular aspects of the technology continue to be made. Tremendous progress has been made in recent years regarding the genetic bases underlying both *in vitro* and *in situ* plant morphogenesis, stimulated by progress in functional genomics research.

Morphogenesis involves new structure and organization that involves physiology and morphology aspects. Thus, plant morphogenesis can partly be referred as a regeneration system for adventitious shoots, shoot buds, micro shoots and also somatic embryos (embroids). The two primary morphogenic pathways leading to whole plant regeneration, which is a perquisite for most plant breeding, genetic and transgenic applications of *in vitro* biology, involve either somatic embryogenesis or shoot organogenesis followed by root organogenesis. Both developmental pathways can occur either directly without a callus intermediate stage, termed adventitious or indirectly following an unorganized callus stage, termed *de novo* (Gamborg and Phillips, 1995).

Few plant species have been shown to regenerate by both organogenic and somatic embryogenic pathways, but many plant species can regenerate by one or the other of these pathways.

Organogenesis can occur in two ways which are direct and indirect. Direct organogenesis happens when new plant organs such as shoots, roots and somatic embryos were formed directly from *in vitro* cultured explants. While through indirect organogenesis, plant organs are formed from unorganized cells (callus). In tissue culture, formation of shoots (caulogenesis) and roots (rhizogenesis) can be induced separately. Somatic embryos or embryoids can be induced from vegetative tissues. This process is known as somatic embryogenesis. Teo (1992) defined the formation of shoots or roots or other organs that are developed from abnormal position such as shoots that are formed from roots as adventitious. The formation of adventitious shoots and roots are commonly induced from various types of explants *in vitro*.

Somatic embryogenesis may be the best example of totipotency expressed among a large number of plants (Thorpe, 2000). Various culture treatments can be manipulated to optimize the frequency and morphological quality of somatic embryos, which are bipolar structures containing both shoot and root apices and developing in a manner parallel to that of zygotic embryos. Typical treatment factors include the plant growth regulator sources and concentrations, choice of explants, nutrient medium composition, culture environment and osmotic potential. Many of these factors have to be adjusted (e.g., carbohydrate and nitrogen sources) or completely changed (e.g. withdrawal or

reduction in auxin signal) during maturation of somatic embryos, during which time they become competent for conversion into plantlets (Thorpe, 2000).

In plant tissue culture, the process in which a complete plant is formed from a small piece of tissue is also known as micropropagation. Micropropagation is the true-to-type propagation of a selected genotype using *in vitro* culture techniques. Murashige (1974 a, b) proposed that there are three stages protocol for micropropagation of plants. However, there are five stages that need to be accomplished in order to ensure the success of micropropagation process. The preparative stage is important and crucial (Debergh and Maene, 1981) in the development of a reliable and repeatable microporpagation scheme. The establishment of axenic and viable cultures is the primary purpose of the whole system. Ploriferation of cultured cells is another crucial aspect in determining the response of cultured cells. The next important factor is the formation of complete plantlets from the proliferation of the cultured cells and finally, the establishments of the plantlets from *in vitro* system to the greenhouse or soil and obtaining the true-to-type plants.

Many of the same culture factors for somatic embryogenesis are also manipulated to induce and optimize organogenesis, but often the factors are manipulated in many different ways (Joy and Thorpe, 1999). For example, high auxin is usually important to induce somatic embryogenesis, whereas high cytokinin to auxin ratio (or high cytokinin with no auxin) is typically required to induce shoot organogenesis. Root initiation also requires a moderate to high auxin signal (Gamborg and Phillips, 1995). Because

regenerated organs are unipolar, two distinct organogenic inductions are required to regenerate a whole plant, one to induce shoots and the other one to induce roots. In contrast, bipolar somatic embryos are induced by a single induction signal (Phillips, 2004).

The significance of micropropagation will only be realized when suitable starting material is used. The choice of stock material cannot be done indiscriminately. For most ornamentals and for fruit production, the starting material is an elite plant, selected for some particular phenotypic characteristics. In forestry and horticulture production, the elite material will either be a selected phenotype (a specific plant) or this plant will be the bearer of elite seed (Debergh and Read, 1990) which will be used as starting material (explant).

The preparative stage was mainly introduced to be a remedy for contamination problems. Nurturing mother plants under aseptic conditions can reduce contaminations especially those which are related to fungi (Debergh and Maene, 1981). Nevertheless it is more difficult to interpret the results with respect to bacterial contamination since most often endogenous and exogenous bacteria are difficult to be distinguished (Cassells, 1990). In order to obtain bacteria-free explants, stock plants can be grown in the controlled greenhouse. For tropical and subtropical ornamentals it is advised to maintain a relatively high temperature (25 ^{o}C) and a relatively low humidity (75%). For nursery plants, a plastic greenhouse is favourable but it is not necessary to control temperature and relative humidity.

The preparative stage includes many kinds of factors that make an explant more suitable or reliable to be used as a starting material. The influence of the stock plant on effective micropropagation has been demonstrated by several researchers. The most common manipulated parameters are light, temperature and growth regulators. Controlling the photoperiod in the greenhouse opens possibilities of producing more standardized stock plant throughout the year. This is important for plants which flowering is under photoperiodic control. For instance, *In vitro* culture of *Begonia* was influenced by high temperatures and long days applied to the stock plants (Heide, 1968). Some plants, especially bulbous crops (Van der Linde and Van Aartrijk, 1986) and trees (Evers, 1987) need cold condition to break dormancy. Stimart and Ascher (1981) found that different durations of cold storage at 4 ^{o}C had a strong effect on production of lily bulblets *in vitro*.

Research on the responses of explants *in vitro* and their relationship to stock plant treatments has been limited. Read and Yang (1985) put branches of *Castanea* and *Aesculus* in a solution containing 2% sucrose, 8-hydroxyquinoline citrate (200 mg/l), 100-800 mg/l BAP and 10-1000 mg/l GA_3 to induce shoot formation. This approach has been used to other woody plant species. An effective method for Petunia was to dip the entire leaf in 400 mg/l BAP and further cultured on cytokinin free medium, which was highly successful in promoting shoot formation (Economou and Read, 1980).

In vitro growth and development of a plant is determined by factors such as the genetic constitution of mother plant, types of media and nutrients supplemented to the

culture medium such as the content of water, macro and micro elements and sugar. The role of plant growth regulators or plant hormones is essential in determining the response of *in vitro* cultured plants. The other important factor is the physical and environmental factors which are light, temperature, humidity, pH, size of culture vessels, oxygen (O_2) and carbon dioxide (CO_2) supply. There are also factors that are related to the explant tissues such as the type, age and size of explants used as starting materials, culture period, polarity of explants in the culture medium and also competition between meristems.

The genetic constitution is a crucial factor at every stage in the plant development, it determines if a plant is a monocotyledon or a dicotyledon, which leaf form it has, which temperature is optimal for growth and flowering, flower form and colour and many others. In *Gerbera*, 12 out or 21 genotypes examined gave rise to haploid cells and only six produced shoot (Tosca, *et al*., 1990). Miyoshi and Asakura (1996) reported that 13 out of 17 genotypes produced callus in *G. jamesonii*. The expression of genetic content in plants also depends on physical and chemical conditions which also have to be created *in vitro*. When initiating tissue culture, explants have different ability to response and to form a new organ. Different plant species has different resistance and strength towards sterilization process. The ability to form adventitious shoots and roots also differs.

Most cultured plants are placed in a culture room at a constant temperature. Some plant species need certain temperature for growth. In *Streptocarpus*, the formation of shoots from leaf explant was optimum at 12 ^{o}C and shoot formation was reduced when the temperature was increased to 30 ^{o} C (Teo, 1992). However, normally 25 ^{o}C is fixed

for *in vitro* cultured plants. Humidity is also important to avoid dehydration of the cultured plants. The normal relative humidity for *in vitro* cultured plants is 70% and some plant species need higher humidity especially in the culture container.

There are three light factors that influenced growth and *in vitro* morphogenesis, i.e. photoperiod, wave length and flux density. The effect of light on photosynthesis of cultured plant *in vitro* is less important. Generally, cultures are incubated in the culture room at 500-3000 lux and later the light intensity is increased to 3000-10000 lux. This helps the survival and adaptation of the cultured plants when they are transferred from the culture vessel to the greenhouse.

The development of plant tissue culture is highly related to the chemical constituents which are the growth regulators (Skoog and Miller, 1957). The nutrient ingredients have long been studied using various culture media such as MS (Murashige and Skoog, 1962), LS (Linsmaier and Skoog, 1965), B5 (Gamborg *et al*., 1968), NN (Nitsch and Nitsch, 1969), N6 (Chu *et al.*, 1975), C2D (Chee and Pool, 1987) DKW/Juglans (Driver and Kuniyuki, 1984), McCown woody plant (Lloyd and McCown, 1980) and many others.

Nutrients are essential for the growth and development of the plants. The content of nutrients in the culture medium determines the success of tissue culture. Nutrients are the source of ions that is needed by plants. The addition of nutrients (macro and micro

nutrient) in the culture medium is similar to the usage of nutrients from the soil in the normal planting. Vitamin is needed by plant cells in very small quantity. It helps to enhance the growth of cultured plant. Some plants are able to synthesize endogenous vitamins. Sugar is added to the culture medium and acts as carbon source that provide energy. The most common sugar used in the culture medium is sucrose.

The supplementation of plant growth regulators in the culture medium such as auxin and cytokinin are important in plant tissue culture. *In vitro* growth and development of plant cells is almost impossible without plant growth regulators. However, they are only needed in very small quantities. Whether an auxin or a cytokinin needs to be added to a nutrient medium to obtain a response of cultured tissues, it is completely dependent on the type of explant and the plant species. Explants which produce sufficient endogenous auxin or cytokinin do not need extra exogenous auxin or cytokinin for their growth.

It is important to determine the balance nutrient medium that is suitable for the growth of tissues from a particular plant type (Skoog and Miller, 1957; de Klerk *et al*., 1997). Auxins (IAA, IBA, NAA, 2, 4-D) are often added to the nutrient media. Auxins play an important role in cell elongation and swelling of tissues, cell division (callus formation) and the formation of adventitious roots. Auxin inhibits shoot formation. Usually, root formation occurred with addition of auxin at lower concentration whereas higher concentration of auxin promotes callus formation (Pierik, 1987). Sometimes the addition of auxin resulted in the promotion of seedling growth. NAA promoted root

formation in seedlings of a selection of Bromeliaceae, which resulted in growth stimulation of germinated plants (Pierik *et al*., 1984).

Cytokinins are necessary used to stimulate growth and development of plants. Most commonly used cytokinins are BAP, kinetin and zeatin. Cytokinins usually promote cell division, especially when combined with an auxin. At higher concentration, cytokinin will induce the formation of shoot, however root formation is usually inhibited (Pierik, 1987).

There are also plants which in principle do not need the addition of neither cytokinins nor auxins for the formation of adventitious shoots such as chicory (Pierik, 1966) and *Streptocarpus* (Applegren and Heide, 1972; Rossini and Nitsch, 1966). However, most plants require cytokinin for shoot formation, whereas auxin generally prevents shoot formation (Miller and Skoog, 1953; Paulet, 1965; Nitsch, 1968). It appears that a high cytokinin concentration and a low auxin concentration are very important for shoot formation in many different plant species. For example, in Begonia (Ringe and Nitsch, 1968; Heide, 1965), horseradish (Wurm, 1960; Sastri, 1963), *Atropa belladonna* (Zenkteler, 1971) and cauliflower (Margara, 1969). There is a rare case where plants require exogenous auxin for shoot formation such as in lily (van Aartrijk, 1984) and hyacinth, (Pierik and Steegmans, 1975).

With plants that have a cytokinin and auxin requirement, the cytokinin concentration should be higher that the auxin concentration and the ratio between these two hormones will determine organ formation (Skoog and Tsui, 1948; Miller and Skoog, 1953; Paulet, 1965; Gautheret 1959). In many plants, BAP is the most common and efficient cytokinin in promoting adventitious shoots formation.

In *Dianthus chinensis* L., leaf explants cultured on solid Murashige and Skoog (MS) medium supplemented with 3.0 mg/l BAP and 0.5 mg/l 0r 1.0 mg/l NAA produced adventitious shoot buds. Shoots were rooted on half strength MS medium with 0.05 mg/l IAA (Kantia and Kothari, 2002). Topoonyanont and Dillen (1988) cultured capitulum explant of *G. jamesonii* on half-strength MS basal medium supplemented with 5-15 mg/l BAP and produced eight shoots per explant with 5.0-7.7 mg/l BAP. Huang *et al.* (1987) reported a scheme for commercial multiplication of *G. jamesonii* through shoot tips cultured on a medium containing MS supplemented with 5 mg/l BAP, 0.1 mg/l IAA and 1% agar. In *Cyrtanthus clavatus* and *Cyrtanthus spiralis*, shoot regeneration were observed when bulbs were cultured in liquid MS media supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA. Rooting was observed when the shoots were transferred to 0.1 mg/l NAA. This showed that hormonal requirement affects plant morphogenesis in tissue culture system.

The response of cultured plant differs with the type of culture media used. The absence of gelling agent in culture media might increase the nutrient intake of the explant since the surface area between explant and media are larger and this allow larger area of

explants to be in contact with the media. Adventitious organogenesis or shoot formation is a preferred system as it enables to retain the clonal fidelity since many ornamental species are cultivars that are propagated for one or more unique characteristics. Also, the propagation rate via organogenesis can be much higher in terms of shoot formation compared to axillary shoot proliferation (Chun, 1993).

There are also some limitations that affect the success of *in vitro* plant tissue culture process. For some tissues, some explants will turn brown and later die. This is due to the phenolic compounds that are produced by damaged excised tissues. Another problem that is usually faced by *in vitro* micropropagation is abnormalities of regenerated plants. Necrosis and vitrification are two common situations that portray abnormal morphological characters of cultured plants (Kataeva *et al*., 1991). Necrosis is a physiological problem that occurs during the development and rooting of plantlet when the shoots were transferred to a cytokinin-free media (Kataeva, 1986).

Necrosis can be observed when the shoots turn to yellowish, then brownish and finally it will die. Meanwhile, vitrification is a condition where explants became hyperhydrated and looks glassy and translucent (Teo, 1992). Some of the morphology, anatomy, physiology and metabolic disoders of the *in vitro* cultured shoots that differ from leaves produced *ex vitro* might be considered to be precursors of the vitrified condition (Ziv and Ariel, 1992).

In culture medium, explants are either placed on top of the medium or submerged in it. In liquid cultures, vitrification was observed in several plant species even when the shoot was not submerged in liquid shake cultures and exposed to the gaseous phase of the culture on a physical support (filter paper bridges or other support (Ziv, 1986; Ziv *et al*., 1987a, b). Increasing the agar concentration may reduce vitrification but very often also lowered propagation ratio (Debergh *et al.*, 1981; Ziv *et al.*, 1983; Hakkart and Versluijs, 1983; von Arnold and Eriksson, 1984). Media that is rich in nutrients such as MS (Murashige and Skoog, 1962) were shown to promote vitrification in some plant species (Paques and Boxus, 1987).

In addition to the physical state of the medium, various dissolved constituents may affect plant morphogenesis. Water availability in the culture is determined by the water potential and the matrix potential, both of which are regulated by solutes and gelling agent. Sucrose is the main carbohydrate supplied *in vitro*. In carnation, shoot apices developed fewer vitrified leaves on media with sucrose higher than 3% (Ziv *et al.*, 1983). The content of growth regulators in the culture media also believed to cause vitrification. Leshem (1983) suggested that imbalance of auxins and cytokinins induced vitrification. In melon, cytokinin also induced vitrification, which could be reversed by reducing the cytokinin level (Leshem *et al.*, 1988). The nature of abnormal morphogenesis *in vitro* (Gaspar *et al.*, 1987) emphasizes the need for the optimization of plant culture conditions *in vitro*.

Besides organogenesis, there is also another process for *in vitro* propagation which is known as somatic embryogenesis. Somatic embryogenesis is a process which forms structures containing a shoot and root connected by a closed vascular system directly analogous to zygotic embryos in regards to histology, physiology and biochemistry (Redenbaugh, 1993). According to Terzi and Loschiavo (1990), somatic embryogenesis has been described for more than a hundred plant species. Somatic embryogenesis has been divided into 4 different stages which are the induction, early development, maturation of embryoid and germination (Ammirato, 1987).

Induction of somatic embryogenesis occurs in 2 different pathways, direct embryogenesis and indirect embryogenesis. Direct embryogenesis is originated from somatic cells while indirect embryogenesis is induced from callus (Donneley and Vivader, 1988). Both types of somatic embryogenesis are important in propagating true-to-type plants. Embryogenic cells indirectly formed when meristematic cells from vegetative part of plants were induced to divide and develop to form globular clumps of cells in the callus. The globular somatic embryo structure will later turn into heart and torpedo shaped somatic embryos before converting to cotyledon and finally form shoots and further develop into new plants (Raven and Johnson, 2001).

The most important parameters that determine the occurrence of somatic embryogenesis are explants type, the developmental stage of the explants and the interaction between explants and growth hormones. Murashige and Skoog (1962) basal medium, often with half strength major salts have been utilized for most species.

Exogenously supplied growth regulators are essential for the process of somatic embryogenesis (Ammirato, 1983). In general, the presence of auxins or substances with auxin activity is necessary for the induction and proliferation of cells that later will differentiate into somatic embryos (De Jong *et al.*, 1993; Michalczuk *et al.*, 1992). The use of 2, 4-D or other synthetic auxins, e.g. dicamba has been used, usually alone or with combination of a cytokinin like BAP, 2iP or Kinetin. Although 1.0-2.0 mg/l 2, 4-D is normally used, much higher levels have been necessary with palms, i.e. 100 mg/l (Reynolds & Murashige, 1979) due to the presence of activated charcoal in the induction medium. Culture initiation, somatic embryo maturation and germination occur on solidified medium.

Maintenance of ploriferating embryogenic cultures is more efficient in suspension culture supplemented with 1 mg/l 2, 4-D or a mixture of auxin and cytokinin (Kochba and Spiegel-Roy, 1977). Somatic embryos cannot germinate normally unless they are fully matured (DeWald *et al.*, 1989, Wang and Janick, 1984). For example, somatic embryogenesis from leaf and internode matured explants of *Paulownia elongata* was successfully achieved when explants were cultured on MS medium supplemented with 10 mg/l TDZ and 500 mg/l casein hydrolysate. Somatic embryos of *Stevia rebaudiana* were induced when leaf explants (matured tissues) were cultured on MS media with 45 – 113 mg/l 2, 4-D and 4 mg/l BAP in a high (120g/l) sucrose concentration (Bespalhok *et al.*, 1993).

The embryogenic cells from which embryos are derived show common features, which are characteristic of dividing cells. These include, small size, dense cytoplasmic contents, large nuclei with prominent enlarged nucleoli, small vacuoles and a profusion starch grains (Williams and Maheswaran, 1986). In order to germinate, embryos must have functional shoot and root apices which are capable of meristematic growth (Merkle *et al.*, 1990). To ensure the production of somatic embryos are practically applicable, an efficient delivery system must be available whereby the somatic embryos are able to be delivered effectively, survive, develop and finally germinate successfully.

Therefore, somatic embryos formed *in vitro* are coated with a special encapsulation matrix and this is also known as synthetic seeds or artificial seeds (Demarly, 1986; Redenbaugh *et al.*, 1986). Synthetic seed production is an applied technology which capitalizes on the capacity for plant multiplication. Artificially encapsulated somatic embryos can be sown under *in vitro* or *ex vitro* conditions, producing uniform clones (Aitken-christie at al., 1995).

The encapsulation technique was first introduced by Murashige (1979). A gel containing nutrients consists of sodium alginate salt is coated around the somatic embryo and acts as artificial endosperm. This artificial endosperm acts as food storage. Apart from containing nutrients and carbon source, growth hormones, pesticides and fungicides can be added to the encapsulation matrix to help the germination of the synthetic seeds and also during transplantation of the plantlets to the green house. Encapsulation matrix also helps to protect the propagules during storage of the synthetic seeds. Redenbaugh et.

al. (1986) stated that the main regulation to form synthetic seed is to use somatic embryos as propagules. Encapsulation of somatic embryos has widely been used for the aim of synthetic seed production.

Besides somatic embryo, other types of explants from various vegetative parts can also be used as propagules for the encapsulation process. Synthetic seeds were successfully produced from encapsulation of micro shoots, shoot bud, protocorm like bodies and nodules. In recent years, synthetic seeds encapsulation techniques have been extended to non-embryogenic *in vitro* derived explants (Muruyama *et al.*, 1997; Standardi and Piccioni, 1998; Soneji *et al.*, 2002; Danso and Ford-Lloyd, 2003; Chand and Singh, 2004; Tsvetkov and Hausman, 2005) for some specific applications. The encapsulated shoot tip of potatoes has been grown in the field and showed good harvests (Nyende *et al.*, 2005). Non-embryogenic artificial seeds are comparatively inexpensive and easy to handle, transport and grow (Danso and Ford-Floyd, 2003). Synthetic seeds of *Pogonatherum paniceum* were produced when shoot buds were encapsulated in MS medium containing 3% sodium alginate, 3% sucrose with 1% activated carbon (Wang *et al.*, 2007). The encapsulated shoot buds can germinate and grow into complete plants in both aseptic and non-aseptic conditions and have high survival rate.

There are several factors that influence germination of synthetic seeds such as the concentration of encapsulation matrix or sodium alginate used for encapsulation, type and culture condition during germination, nutrient content in the encapsulation matrix and carbohydrate source that is supplemented to sodium alginate capsules. The most

important factor is probably the quality of propagule used to make the synthetic seed. The success of synthetic seed germination also depends on the suitable storage period. Encapsulated propagules have viability to survive for a longer storage period. According to Redenbaugh *et al.*, (1988) most crops can be stored from 6 to 12 months prior to germination and further growth. Synthetic seeds can be stored at 4 ^{o}C for a minimum of 7 days (Redenbaugh *et al.*, 1987) and to a maximum period of 180 days in *Valeriana wallichii* DC. (Mathur *et al.*, 1989). However, the germination quality depends on many factors such as the quality of propagules of the plant species that is used to be encapsulated.

Janeiro *et al.*, (1997) reported that production of synthetic seeds of *Camellia japonica* was successfully achieved when somatic embryos were encapsulated using Ca-free liquid MS medium added with 3% sodium alginate, 3% sucrose with the addition of 5.0 mg/l GA_3 and 18 mg/l IAA. Encapsulation of camellia somatic embryos caused no reduction in *in vitro* complete germination rate as long as the encapsulation matrix was added with nutrients and growth regulators.

There are many advantages of synthetic seed production. Synthetic seed technology has large potential to contribute to plant micropropagation. Production of synthetic seeds is important to seedless plants or plants with difficulties to produce seeds, limited number of seeds, minute seed size with minimal endosperm, seeds with low viability, plants that need a long period to produce seeds or have long dormancy period

and plants that produces seeds that is unmanageable and difficult to transport from one place to another (due to unsuitable seed texture).

In addition, synthetic seed production is important to overcome plants with germination difficulties due to unbreakable and hard seed coat, thick and stiff endosperm, dormant seeds and many others. Synthetic seed is also suitable for hybrid plants and polyploidy plants. Thus, synthetic seed is introduced to overcome problems that are associated with plant propagation. Production of synthetic seed is also another way for germplasm storage where only a minimal size is required for a number of plant species. This can be done in laboratory scale without depending on the weather and outside environment.

Through synthetic seed technology, enough supply of seeds can be produced. The synthetic seed technology is a high volume, low cost production technology (Ghosh and Sen, 1994). Synthetic seed is easy to handle while in storage, easy to transport and has potential for long term storage without losing viability. Furthermore, the clonal nature of the resulting plants can be maintained. The synthetic seeds would also be a channel for new plant lines produced through biotechnological advances to be delivered directly to the green house or field and allow economical mass propagation of elite plant varieties.

However, *in vitro* propagation may lead to genetic variation and mutation. The variation can be observed through the new plant phenotype produced. Genetic variation is

used to describe heritable variation that is sexually transmitted to the progeny of plants regenerated from cultured cells while mutant is usually for special cases in which a trait is transmitted meiotically according to well established laws of inheritance. The term variant is used when the nature of the heritable changes is not known (Meins, 1983).

In plants, somaclonal variations occur when genetic variation is observed in regenerated *in vitro* plantlets (Scowcroft, 1985). Somaclonal variation can also occur during *in vivo* propagation (Orton, 1984). In *Saintpaulia ionantha* and *Begonia x hiemalis*, somaclonal variation may arise due to adventitious shoot formation *in vivo* or *in vitro*. The chances of mutations are increased when cytokinin are added in the culture medium (Scowcroft, 1985; Ando *et al.*, 1986). Pierik (1987) stated that somaclonal variation increase as the increase of genetic variation in higher plant *in vitro*. Somaclonal variation can occur in regenerated plants through totipotent cells and callus that shows chromosome variation and this allow the production of new hybrid. Variation that occurs naturally usually related to plant tissue culture. There are a few types of variation that usually occur which are the morphological and physiological changes of undifferentiated callus, changes in organogenesis that exhibits changes of plantlets and chromosomes (Green, 1977).

Besides the effect of radiation, mutation can also occur in newly regenerated plantlets *in vitro* and *in vivo* due to culture procedures, mutation agents, chemicals etc. The changes can be observed through the new phenotype produced. Mutation in plants normally arises when plants are exposed to radiation at certain doses (Mak *et al.*, 1990).

Preliminary tests showed that radiation affects the morphological characters of *in vitro* and *in vivo* plants. Mutations often appear during callus, cell and protoplast cultures (Bayliss, 1980; D'Amato, 1978).

The use of plant growth regulators such as 2, 4-D, NAA and synthetic cytokinins lead to a large increase in the number of mutations. Kinetin appears to induce cell division in cells which are naturally polyploidy (D'Amato, 1977). The use of BAP at high concentration strongly induced somaclonal variation in tobacco (Evans and Sharp, 1986). Starting materials (source of explants) also has an important effect on the production of mutations. Polyploids species exhibit chromosomal abnormalities more often that diploids (Scowcroft, 1985). Repeated number of *in vitro* subcultures also increases the chance of mutations. This can be observed in the protocorm culture of orchids, axillary and adventitious shoot formation and callus, suspension and single cell cultures.

Mutation induction can be induced by chemicals like ethylmethanesulphonate and colchicine or through physical mutagens which is irradiation by gamma ray, X-ray and many others. The most common method to induce mutation is irradiation (Broertjes, 1981). Roest and Bokelman (1976) used irradiation treatment and *in vitro* culture technique to induce flower colour mutations in chrysanthemum. Roest (1980) and Broertjes and van Harten (1978) proposed that mutation induction *in vitro* can be an advantage to plant breeder. Mutations by irradiation can be obtained (in terms of flower, sizes, flower colours and many other characteristics) while other desirable characteristics are retained.

Most plant species has different radiation reaction level. Further research is needed to identify irradiation doses that could induce mutation. The production of mutants is important in somatic hybridization. Hybridization is a technique in which plant clones are produced with high quality and of agricultural importance. Plants with high resistance against diseases and produced early flowering can be produced. Today, induced mutation breeding by nuclear technique has been introduced to improve hybridization technique.

Induced mutation breeding is done through irradiation technology in which plants are exposed to irradiation sources like gamma ray and X-ray. This technique has proven to affect genetic variation on plants as the morphological characters were changed. The characters of irradiated plants can be observed morphologically through the differentiation on the leaf size and colour, flower size and colour, height and growth of plants and many other characters.

Plant tissue culture process is considered successfull when the micropropagated plants are acclimatized and transferred to the greenhouse (George and Sherrington, 1984). Acclimatization is a natural adaptation process for most *in vitro* micropropagated plant species towards the environment (Preece and Sutter, 1991). Micropropagated plants usually showed low survival rates when acclimatized due to the lost of water, inefficient stomata functions, poorly developed cuticle wax on leaves produced and many other inefficiency (Majada *et al.*, 1998). Normally, micropropagated plants *in vitro* could not survive the stress when transferred to a different environment.

There are other factors that influence the survival of acclimatized micropropagated plants. The relative humidity in the culture vessels are higher compared to normal environment. Thus, the plant should be given time to get use and adapt to the *in vivo* condition. Plantlets need to be placed in the culture room for a certain time period (up to 3 weeks) before they are ready to be transferred to the green house. According to Ziv (1986), the culture vessels need to be opened a few days before *in vitro* plantlets are transferred to soil and outside environment. This is to increase the cuticle wax on plant epidermis. Thus, water loss through evaporation can be reduced. Acclimatization can take place by allowing the *in vitro* plants to gradually get used to a lower relative humidity environment. Plant propagation via tissue culture is a success when the *in vitro* plantlets survived during the acclimatization process.

Plant tissue culture has made significant contribution towards plant breeding. To this date, tissue culture technique has brought many advantages especially towards horticulture and agriculture industries. Plants can be propagated through vegetative parts and not only depending on seed production. Plant organs such as leaves, stem and root can be used as explants for micropropagation. Micropropagation is essential for propagation of seedless plants. Other plant parts and organs can be used for alternative starting materials.

Disease-free plants can be produced through tissue culture technique. Meristematic parts of plants such as shoot tips are usually protected and free from infections. Thus this part is usually preferred to be used as source of explant. Tissue

culture technique is strictly practiced under sterile and aseptic condition. Thus, the infection of microorganisms can be prevented to some extend. Additionally, in plant tissue culture, the starting material or source of explant is usually selected from a healthy and vigorous mother plant. This is to ensure the micropropagated plants have the same quality and standard as the mother plant.

Through plant tissue culture, micropropagation of plants with limited number of seeds and also seeds with long dormancy period can be overcome through embryo culture. The production of synthetic seeds also helped in micropropagation of seedless plants and plants that faces problems in producing healthy and viable seeds. Synthetic seeds produced through the encapsulation of somatic embryo or other vegetative parts of plants can be stored and germinated whenever needed.

Another advantage of plant tissue culture is production of haploid plants. Haploid plants can be produced through the culture of anther or microspores. Production of haploid plants is important in detecting mutants in plants. Mutants usually are hidden when in heterozygous condition. Triploid plants can also be produced from endosperm tissues. Through plant tissue culture, only one step is needed to produce triploid plant compared to conventional method where hybridization needs to be done between tetraploid and diploid plants and this is very time consuming.

Plant tissue culture also allows germplasm storage where a collection of vigorous, healthy and economically important plant stocks can be stored. Germplasm storage is very important for conservation of endangered plant species, hybrid plants obtained from protoplast fusion and many other rare plant species. Plant genetic manipulation and improvement through genetic engineering can be constructed where quality genetic contents can be transferred. Certain genes that control a specific character can be identified via genetic engineering.

Another important aspect of plant tissue culture is that valuable and economically important chemical compounds can be extracted from callus cultures. Large scale compound production can be produced when callus are cultured in bioreactor. Through this method, conservation of intact plants can be maintained and harvesting of *in vivo* plants can be limited since callus culture is induced from only a small plant tissue.

In the past decade, many of the technical improvements resulting in improved *in vitro* plant regeneration system have been related to manipulation of the gaseous and/or physical environment of the cultures. Culture plant tissues are known to interact with the culture medium and gaseous environment. Forced ventilation and use of ventilated culture vessels, for example, have facilitated optimization of *in vitro* morphogenesis systems and high CO_2 treatments have permitted establishment of photoautotrophic cultures (Buddendorf-Joosten and Woltering, 1994). Control of the amount of ethylene released by the cultured tissues into the head space of the culture vessel, or alternatively,

inhibition of ethylene synthesis or action have led to improved morphogenic responses (Kumar *et al.*, 1998).

Efforts to improve bioreactor designs to facilitate economical large-scale production of plants of plant products have continued. The importance that must be addressed with bioreactors designs for plant cell and tissue growth include aeration and minimization of shear damage. Advances in automation and computer controls have rendered bioreactor performance more reliable (Paek *et al.*, 2001). One of the most exciting developments in bioreactor designs has been temporary immersion system, which alternates immersion of the plant tissues in the liquid culture medium with exposure to the air space at timed intervals (Etienne and Bethouly, 2002). Temporary immersion bioreactors have been demonstrated to improve yields of shoot proliferation cultures, microtubers and somatic embryos, as well as improve the quality and vigor of the propagules with reduced frequencies of abnormalities and hyperhydricity.

Another interesting development in *in vitro* culture is the use of perfluorochemicals and commercially stabilized bovine hemoglobin as gas carriers to enhance cell performance in liquid culture systems such as bioreactors. Perfluorochemicals are recyclable which can be used to deliver gases, then recovered from the culture and recharged. These gas carriers have been shown to improve cell division rates, stimulate biomass production, improve yields of cellular products and enhance morphogenic totipotency (Lowe *et al.*, 1998, 2003). Another technical innovation with more of a physical impact on the culture environment is the use of semi-

permeable cellulose acetate membranes to enhance citrus somatic embryogenesis and particularly to normalize somatic embryo development (Niedz *et al.*, 2002).

Plant tissue culture is a powerful tool of plant propagation since it does not depend on the climate and weather to produce new plants. Under controlled conditions, *in vitro* plantlets can be obtained at any time. This shows that plant tissue culture is a convenient and cost effective method for *in vitro* micropropagation and mass propagation. Besides, plant tissue culture technique is also a very environmental friendly technique where production of hundreds of new plants can be obtained from small pieces of plant tissues.

Basic research has begun to dissect the complex genetic pathways involved in various aspects of plant morphogenesis, including all of the major pathways leading to *in vitro* plant regeneration. A number of candidate genes are being identified to be expressed transgenically to enhance or even to initiate plant regeneration from cultured cells or tissues. Such genes are being explored for potential use in developing marker-free transgenic systems as well as to potentially enhance the frequencies of transgenic plant recovery (Zuo *et al.*, 2002). Advances in specific culture systems such as thin cell layers (Nhut *et al.*, 2003) offer the prospects of extending more efficient *in vitro* plant regeneration techniques to previously recalcitrant crops and of developing more efficient genetic transformation methods. Such advances in controlling *in vitro* morphogenesis should play important roles at the applied level in developing new crop cultivars and

reducing the cost of micropropagation and in furthering basic research in the area of functional genomics by testing of transgenes in a wider array of plant species.

1.2 INTRODUCTION TO THE FAMILY ASTEREACEAE (COMPOSITAE)

The family Asteraceae is also known as the sunflower family. There are more than 1550 genera and 23,000 plant species that belong to this family including trees as high as 30 metres tall and small herbs that is about 1 cm high. Flowering heads, or capitula that appears in different sizes, shapes, and colours is a common structure to the giant heads of cultivated sunflower. Asteraceae are found everywhere in every continent except in Antarctica. They are mostly found in temperate regions of the world or in cooler habitats. They are neither common in tropical rainforest nor in the hot lowlands.

Vegetative features in asteraceae family differ significantly. Some of the members in this family are large trees, such as *Vernonia arborea* or *Dasyphyllum excelsum* that reach more than 30 meters long and have trunks that is more than 1 meter in diameter. There are also some varieties of shrubs such as *Chrysothamnus*. Some are well armed such as *Chuquiraga* or *Barnadesia*. Some are rosette trees (Dendroenecio). Most of the members of the family are perennial, herbs that inhabit numerous ecological zones, with a preference towards temperate region. Some species from this family are typical upright sunflowers, about 1-3 meters tall, whereas others are of reduced size down to being flat on the ground as in *Hypochaeris sessiliflora* of the Ecuadorean alpine zone. One of the

smallest genera is *Mnioides* of the Peruvian Andes, which measures only a few centimeters tall. There are even a few aquatic Asteraceae such as *Helianthus angustifolius* (Hutchinson, 1916).

Leaves vary widely within the family. Some leaves are very large, for example, nearly 1 meter long in *Clibadium grandifolium* (Heliantheae). Others are small, being almost nonexistent as in *Baccharis sagittata* in which the stems are green, photosynthetic and conspicuously winged and have taken over the function of leaves. Some leaves are very spiny such as *Carduus* spp. Other leaves are covered with varying degrees of indumentum, hairs of all densities, lengths and colours. Many species of well known genus are attractive for their interesting hairy leaf surfaces, making desirable horticultural plantings.

The basic reproductive unit of asteraceae is the head or capitulum. This is a cluster of flowers or an inflorescence. The flowers are arranged sessile (unstalked) on the point of attachment (to the receptacle). Heads of asteraceae are remarkable in variation of their structures and these modifications often have taxonomic importance at different levels in the hierarchy.

*Gerbera*s, chrysanthemums, dahlias, ageratum, lettuce, coreopsis, chicory, endive, sunflowers, ragweed and thistles all belong to Asteraceae family. It is relatively easy to summarize the geographic distribution of Asteraceae. The family occurs on every

continent except Antarctica where there are only two angiosperms, *Colobanthus quitensis* (Caryophyllaceae) and *Deschampsia Antarctica* (Poaceae) (Moore, 1983). This means that the more than 23,000 species of the family have been successfully dispersing to new habitats and becoming established. Within particular continents, Asteraceae occur nearly everywhere in just about every conceivable habitat. However, the family is more abundant in temperate areas or in cooler, higher elevation regions of the tropics.

The International Code of Botanical Nomenclature (Greuter *et al.*, 2000) allows two acceptable names for eight plant families, including Asteraceae. The name Compositae is a very old descriptive term referring to the head inflorescence. Although it is the oldest name, it is not formed correctly with –aceae ending. Hence Asteraceae has been conserved, whereas the older name, Compositae, is sanctioned as an alternative (*nom. alt.*). Both terms, Asteraceae and Compositae are regarded as acceptable.

1.2.1 GENUS *GERBERA* (AFRICAN DAISY)

Gerbera is a flowering perennial plant which belongs to the Asteraceae family. It is a very well known plant and useful as cut flowers, pot crops and bedding plants. There are more than 80 species under this genus. Some of the species are;

1. *Gerbera ambigua*
2. *Gerbera anandria*
3. *Gerbera candollei*
4. *Gerbera chilensis*

5. *Gerbera galpini*
6. *Gerbera jamesonii*
7. *Gerbera lanuginose*
8. *Gerbera nivea*
9. *Gerbera speciosa*
10. *Gerbera tomentosa*
11. *Gerbera viridifolia*
12. *Gerbera wrightii*

Gerbera jamesonii produce leafless flower stalk with beautiful flowers that comes in various colours such as red, yellow, pink, orange or white. Robert jameson, a Scotsman, first discovered *Gerbera* daisies while operating a gold mine near Barberton in the Transvaal area of South Africa in 1880. He donated the plants to the Durban Botanical Gardens, and the curator of the Garden, John Medley Wood sent specimen to Harry Bolus in Cape Town, South Africa for identification. Bolus sent specimens to the Royal Botanical Gardens at Kew in England, with suggested scientific name, *Gerbera jamesonii*.

Gerbera jamesonii is one of the most popular plants from Asteraceae family and could be easily obtained in many countries. It is popularly known as 'Barberton daisy' or 'Transvaal daisy'. *Gerbera*s are mostly found in temperate countries. However, through researches that have been done, it is proven that this plant could be successfully planted in tropical countries such as Malaysia.

Gerbera jamesonii starts to produce flowers when the plant reaches the height of 12 inches or more. It has no leaf stalk. The leaves are arranged to form 'rosette' and supported by petiole. The length of the leaves varies between 5 to 10 inches and width ranging from 2 to 4 inches. *Gerbera* has simple leaf with 'oblanceolate' or sometimes 'ovate' shape. The upper part of the leaf is 'acute' which is essentially straight or slightly convex and the lower part of the leaf (near the petiole), is 'hastate'. Leave margin of this plant are in 'parted' and sometimes in 'cleft' shape. The leaf structure is soft and hairy. Adaxial part of the leaf is usually dark green and the abaxial part looks lighter green or pale green.

Normally, *G. jamesonii* flowers often measure around 7 inches. The flower usually located in the middle of the leaves rosette at the leaves apex. At the end of a flowering period, the plant will go through a conditioning and rest phase in which it will go through another flowering cycle in a few weeks or even a few months time. The classifications of *G. jamesonii* are as below;

Kingdom : Plantae

Subkingdom : Tracheobionta – vascular plants

Division : Magnoliophyta – angiosperms

Class : Magnoliopsida – dicotyledons

Subclass : Asteridae

Order : Asterales

Family : Asteraceae

Subfamily : Mutisioideae

Tribe : Mutisieae

Genus : *Gerbera*

Species : *Gerbera jamesonii* Bolus ex Hook f.

Besides being used as ornamental plants and for decorative purposes, *G. jamesonii* also has medicinal values. It is being used as Chinese traditional medicine to treat flu, cough and rheumatism (Ye *et al.*, 1990). The overall plant could be used for this purpose. This plant is also identified to have the properties to remove toxic compounds from the environment.

Some research has proven that *G. jamesonii* is one of the decorative plants that could overcome the increase of pollutants in buildings. It could absorb pollutant gases and at the same time cleaned the air. Besides *G. jamesonii*, other ornamental plants that have this unique character are *Chamaedorea seifritzii*, *Aglaonema modestum*, *Chrysanthemum morifolium* and *Dracaena marginata* (Wolverton *et al.*, 1984).

Gerbera jamesonii can be considered as an ornamental plant which has very high commercial values. It is the fifth most used cut flower in the world (after rose, carnation, chrysanthemum and tulip). Not only producing beautiful flowers, this plant also has good medicinal values and able to reduce pollutants from the air we breathe.

Thus, more research needs to be done in order to produce this plant in large scale with high quality and the ability to be produced throughout the year. An alternative method to propagate this plant without worrying about the weather and environmental stresses is through plant tissue culture. Since the plant is mostly suitable to be planted in temperate regions, thus, more research need to be done in order to obtain *Gerbera* plants that could be grown successfully in Malaysia with very high economical values and more hybrids of this species could be produced.

1.3 RESEARCH OBJECTIVES

Gerbera jamesonii (Figures 1.1-1.3) is a beautiful temperate ornamental plant with very high commercial value. Through plant tissue culture, this plant can be mass propagated and grown in tropical countries. Micropropagation of *G. jamesonii* is very important since the plant is of high demand.

The main objective of the present study was to establish *in vitro* propagation of *G. jamesonii* Bolus ex. Hook f. The plant species used was the natural breed with pink colour flowers. Several factors affecting micropropagation of the plant to achieve complete plant regeneration such as media, growth hormones and substrates, explants potentials, physical factors and many others were investigated.

Regeneration potentials of *in vitro* propagation from various types of explants (leaf and petiole) and the effect of polarity of explant were investigated. Two-month-old

plantlets obtained from germination of aseptic seedlings were used as sources of explants. The most responsive explant with suitable polarity was used for subsequent experiments.

The effect of growth hormones (balance between auxin and cytokinin) and media components on morphogenesis response *in vitro* were also investigated. Different combinations of auxin (NAA) and cytokinin (BAP) at various concentrations were used for this purpose. Other types of auxin (IAA, 2, 4-D and IBA) and cytokinin (zeatin, kinetin and 2iP) at 2.0 mg/l were tested to study *in vitro* regeneration of *G. jamesonii*. The purpose of this experiment was to compare the effect of combination of BAP and NAA and other types of auxins and cytokinins. The optimum condition and growth hormone for *in vitro* regeneration was identified.

Besides shoot regeneration, callus formation was also studied. Plant growth hormones that could induce callus formation such as 2, 4-D and BAP were used in this study. The optimum condition for callus formation was observed. Preliminary test of secondary metabolite contents in the callus was studied using thin layer chromatography (TLC).

The subsequent experiment was to induce somatic embryogenesis in *G. jamesonii*. Embryogenic and non-embryogenic callus were induced and identified. Through double staining method, embryogenic callus could be distinguished from non-embryogenic callus. Somatic embryogenesis was then induced from embryogenic callus through a series of experiments. Solid and suspension cultures were used. Suspension cultures were

mainly used to induce somatic embryos from the embryogenic callus. The structures of somatic embryos from globular, heart, torpedo and cotyledonary phases of somatic embryos were observed through microphotography microscope and scanning electron microscope (SEM).

Somatic embryos induced were later used as propagules for the production of synthetic seeds. Micro shoots were also used as sources of explants for synthetic seeds production. Today, combination of tissue culture and encapsulation techniques can be utilized for production of synthetic seeds or artificial seeds. Synthetic seed is an alternative plant breeding method to help overcome problems that are related to plant propagation. Synthetic seeds have the capability to be stored and introduced to germplasm storage. *Gerbera jamesonii* is usually propagated through cuttings and seed germination. However, it is difficult to obtain *Gerbera* seeds in Malaysia and it is highly priced since it has to be imported. Nevertheless, sometimes the seeds obtained has very high dormancy period and is not viable for germination. Thus, through tissue culture, a large numbers of new *Gerbera* plantlets can be obtained when only a small pieces of plant tissue is required for micropropagation. Production of synthetic seeds of *Gerbera* will ensure sufficient supply of the plant and *Gerbera* will readily be propagated when needed. This is indeed a very efficient, cost effective and useful system.

The optimum encapsulation matrix for the production of *Gerbera* synthetic seed was identified. In this study, synthetic seed produced need to be idyllic in size, texture with optimal hardness to allow smooth germination. Experiments to identify the optimum

concentration of sodium alginate ($NaC_6H_7O_6$) for the formation of idyllic beads and calcium chloride ($CaCl_2.H_2O$) as the complexing agent were constructed. Factors affecting germination of synthetic seeds such as type of germinating media, composition of encapsulation matrix and storage period at low temperature (4 ± 1 ^oC) were investigated.

In the following experiment, the focus was on mutation induction through gamma irradiation. Explants were exposed to Gamma irradiation at different doses. The effects of gamma irradiation on morphogenesis and callus induction were studied. Somaclonal variation was checked and recorded in the regenerated plants. Morphological characters between irradiated and non-irradiated plantlets were compared based on plant height, leaf and flower characters. From these experiments, it is hoped that irradiated *in vitro* plantlets and acclimatized *in vitro* plants could produce useful variants.

Finally, an efficient acclimatization system was established for this species. Plantlets obtained from *in vitro* micropropagation, somatic embryo induction, germination of synthetic seeds and irradiated plantlets were transferred to various sowing media or substrates and transplanted to the green house. Macromorphological characters of acclimatized *in vitro* plantlets were observed and compared with intact plants. *Ex-vitro* flowering of acclimatized plantlets was also observed and noted. The success of acclimatization process is an assurance that the whole micropropagation process, from the beginning of selecting the starting materials, initiation of *in vitro* cultures and establishment of an efficient acclimatization system has been achieved. As a result, an

economical and effective mode of micropropagation for *G. jamesonii* Bolus ex. Hook f. was accomplished.

Figure 1.1: Nine-month-old intact plant of *Gerbera jamesonii* Bolus ex. Hook f. with flowers

Figure 1.2: Intact flower of *Gerbera jamesonii* Bolus ex. Hook f. plant

Figure 1.3: Nine-month-old intact plant of *Gerbera jamesonii* Bolus ex. Hook f.