CHAPTER 9

DISCUSSION

The present work deals with morphogenesis and tissue culture studies of *Gerbera jamesonii* Bolus ex. Hook f. The species selected is an attractive flowering plant and has very high commercial values. This plant is a temperate plant and it is difficult to plant them in tropical climate like Malaysia. However, based on the research done in this thesis, it is proven that temperate grown flowering plant has the potential to be planted successfully in Malaysia. The present investigation is divided into several parts or chapters. It comprises the studies of *in vitro* micropropagation, callus induction, somatic embryogenesis, production of synthetic seeds, effects of gamma irradiation and finally acclimatization of *Gerbera* plants to the green house.

Initially in chapter two, the studies of *in vitro* regeneration of *G. jamesonii* Bolus ex. Hook f. was undertaken. Various factors were studied to establish good regeneration system for this species. Factors studied include type of explants, polarity of explant, effect of hormones and physical factors in order to obtain optimum regeneration system for *G. jamesonii*. All experiments used MS (Murashige and Skoog, 1962) media and all cultures were incubated in the culture room at 25 ± 1 ^{o}C with photoperiod of 16 hours light ad 8 hours dark. Thirty replicates were used in all experiments and observations were made every week for 8 weeks. Both leaf and petiole explants of *G. jamesonii* successfully regenerated shoots when cultured on MS medium supplemented with various hormones. MS basal medium was not suitable in induction of shoots. Leaf explant showed optimum response of shoot formation when abaxial part of the leaf was

cultured onto the culture medium with 25.0 ± 1.3% and 2.6 ± 0.5 shoots per explant. Petiole explants produced highest shoot formation when cultured horizontally on the culture medium with 90.0 ± 0.5% and 8.1 ± 0.4 shoots per explant (Table 2.1). Petiole explant showed lower shoots formation when cultured vertically. Position and polarities of explants that gave optimum results were further tested in subsequent experiments.

Direct and indirect organogenesis of *G. jamesonii* were achieved when leaf and petiole explants were cultured on MS media supplemented with BAP and NAA at various concentrations (Table 2.2). Results showed that petiole explant produced the highest shoot regeneration (94.3 ± 2.5%) when explant was cultured on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA with formation of 9.3 ± 0.6 shoots per explant. Leaf explant produced the highest shoot regeneration (25.0 ± 1.3%) when explant was cultured on MS medium fortified with 1.0 mg/l BAP and 1.0 mg/l NAA. From the results, regeneration of shoots was better achieved from petiole explant. Further experiments were done using other auxins and cytokinins. Petiole explant was chosen as source of explant as it showed optimum response in the previous experiments. Results in Table 2.3 showed that MS media supplemented with combination of 2.0 mg/l Zeatin and 0.5 mg/l IBA gave the highest shoot regeneration at 83.7 ± 1.5% with 7.4 ± 0.9 shoots per explant. MS basal media was identified as the optimum media in rooting of *Gerbera* shoots.

Different methods of *in vitro* multiplication and regeneration of *Gerbera* have been previously described including adventitious root formation and callus induction

from young leaves (Pierik and Segers, 1972), direct adventitious shoot formation from excised capitulum explants (Pierik *et al.*, 1973) and from isolated shoot tips (Murashige *et al.*, 1974). Askari and Fotouhi (2009) reported that young capitulum explant was used to obtain shoot regeneration of *Gerbera* cultivar 'Jaimy'. Explants were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 0.5 mg/l and 0.1 mg/l TDZ. It was also reported that development of shoot from capitulum explant with involucral bracts only occurred under high cytokinin concentration (Pierik, 1987).

Huettemann and Preece (1993) reported that indirect establishment of *Gerbera* plantlets was achieved using mature and immature capitulum and the influence of BAP and Kinetin on shoot regeneration was reported (Murashige, *et al.*, 1974; Constantinovic and Sandu, 1995). A new method for shoot regeneration of *G. jamesonii* by receptacle Transverse Thin Cell Layer culture supplemented with various concentration of TDZ was also reported (Nhut, *et al.*, 2007).

Ray *et al.* (2005) found that *in vitro* shoot regeneration was achieved when capitulum explants were cultured in culture medium containing 7.0 mg/l BAP and 0.1 mg/l IAA while Purnima and Kothari (2004) obtained shoot regeneration from the same explant when 4.0 mg/l Kinetin and 0.01 mg/l IAA was added in the culture medium. Mandal *et al.* (2002) achieved *in vitro* shoot regeneration from capitulum explant when 10 mg/l BAP was added in the culture medium.

Radojevic *et al.* (1987) initiated adventitious shoots from *Gerbera* cultivar 'Florijn' leaf stalk segments and cultivar 'Claudia' flower segments on MS (Murashige and Skoog, 1962) medium containing 0.2 mg/l kinetin and 0.5 mg/l IAA with the addition of 100 mg/l tyrosine. Jerzy and Lubomski (1991) were the first to demonstrate effective regeneration of adventitious shoots from *in vitro* explants of 28 *Gerbera* cultivars. Shoots were regenerated directly from petioles or from callus obtained at the base of petioles on MS medium supplemented with 3.0 mg/l BAP and 0.5 mg/l IAA. Regeneration was more effective from petiole explants with lamina cultured on medium containing 5.0 mg/l Kinetin than from similar explants excised from rooted shoots.

Reynoird *et al.* (1993) obtained adventitious shoots from leaves of various sizes on medium containing 2.3 mg/l BAP and 0.2 mg/l NAA. Regeneration from mature leaves was promoted when 0.02 mg/l or 0.15 mg/l TDZ was added to the culture medium. 52% shoots were regenerated when petiole explant of *Gerbera* cultivar 'Terra Regina' was cultured on medium supplemented with 1.0 mg/l BAP, 1.0 mg/l zeatin and 0.1 mg/l IAA. Orlikowska *et al.* (1999) obtained 99.6% shoot regeneration from petiole explant of *Gerbera* cultivar 'Mariola' when explant was cultured on modified MS medium supplemented with 0.04 mg/l TDZ and 0.05 mg/l IAA. However, this result was in contrast with the present study where TDZ was found to be unsuitable for regeneration of *G. jamesonii*.

It can be observed that, results obtained in regeneration of shoots varied according to the plant species and the culture environment. Various explants were used for

regeneration purpose of *G. jamesonii*. However, in the present work, capitulum explant was unable to regenerate shoot, instead, only proliferations of callus was obtained. *In vitro* propagation of *Gerbera* was studied by culturing leaf bud explants on a medium containing 1.0 mg/l BAP (Barbosa *et al.*, 1994). Aswath and Choudhary (2002b) reported that a high frequency of shoot organogenesis and plant establishment protocol was developed for *ex vitro* leaf derived callus with 0.4 mg/l BAP and 4.0 mg/l NAA. Meanwhile, Xi and Shi (2003) micropropagated four *Gerbera* cultivars using 0.5-1.0 cm long young leaves with the addition of 1.0 mg/l BAP and 0.1 mg/l NAA in the culture media. In the present study, similar growth hormones were used to obtain shoot organogenesis. MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA was identified as the optimum medium for regeneration of *G. jamesonii*.

Ruffoni and Massabo (1991) indicated that leaf, petiole and shoot apices from *in vitro* plant were able to regenerate some buds. The result obtained was in compliance with the present study where petiole and leaf explants were able to regenerate shoots successfully. Callus induction and subsequent plant regeneration from leaf explant of *G. jamesonii* was observed when explants were cultured on MS medium supplemented with NAA, BAP and IBA in different concentrations (Parthasarathy *et al.*, 1996). Adventitious shoots were observed on MS medium supplemented with 1.0 mg/l NAA and 0.75 mg/l IBA. This result was in contrast to the result obtained in the present study since the addition of NAA or IBA in the culture medium was found to induce formation of root.

Orlikowska *et al.* (1999) stated that the effectiveness of shoot regeneration depended on the cultivar, the sequence of passage on regeneration medium, growth regulators and duration of the induction period. Xu *et al.* (2002) and Kumar *et al.* (2004) regenerated adventitious shoots from petiole and leaf pieces of *G. jamesonii* in a medium supplemented with different concentrations of auxins and cytokinins. Besides using leaf, petiole and capitulum as sources of explants, shoot apices or shoot tips are another alternative that can be used for regeneration and multiplication of *Gerbera* for commercial use. In *Chrysanthemum morifolium*, successful *in vitro* multiplication was achieved on MS medium supplemented with 0.3 mg/l BAP (Shatnawi *et al.*, 2009). Nikam and Shitole (1999) reported that *in vitro* plantlet regeneration of *Carthamus tinctorius* L. were optimized when leaf explants were cultured on MS medium supplemented with 0.1 mg/l kinetin and 0.1 mg/l NAA.

The *in vitro* rooting capacity generally *in vitro* rooting of any plant species such as Rose depends on the interaction of internal and external factors (Hyndman *et al.* 1982). Badzian *et al.* (1991) reported the use of MS medium with major elements reduced to 1/3 to 1/4 strength for root induction. Relatively low salt concentrations in the culture media are known to enhance rooting of micro shoots (Murashige, 1979). Skirvin and Chu (1979) induced rooting of micro shoots of Rose on growth regulators free solidified medium. Sucrose concentration acts as an enhancer of osmotic potential and also plays a vital role in root induction. Lakes and Zimmerman (1990) showed that highest rooting percentage in apple was achieved on a medium with high osmolarity. Rout *et al.* (1990)

reported that rooting of micro shoots of Roses was better on a solid medium as compared to liquid medium.

In *Ocimum basilicum*, a type of herb from Labiatae family, highest frequency and healthy rooting of plantlets were achieved when plantlets were transferred to MS medium containing 0.1 mg/l NAA (Begum *et al.*, 2002). Karam and Al-Majathoub (2000) reported that shoots formed from peduncle and etiolated petiole cultures of *Cyclamen persicum* Mill. were rooted on MS medium containing 1.0 mg/l NAA. The findings are in agreement with those observed in other plant species such as *Adhatoda vasica* (Amin *et al.*, 1997, Azad and Amin, 1998), *Caphaelis ipecacuanha* (Jha and Jha, 1989), *Ocimum sanctum* (Begum *et al.*, 2000), *Plantago ovata* (Wakhlu and Barna, 1989), *Rehum emodi* (Lal and Ahuja, 1989) and *Ruscus hypophyllum* (Jha and Sen 1985). In the present study, rooting of *Gerbera* plantlets were optimum when plantlets were transferred to MS basal medium. However, the addition of NAA in thee culture media also induced rooting of plantlets in this study.

Khosh-Khui and Sink (1982) also reported the effect of different concentrations and combinations of auxins on root formation in *Rosa hybrida* cultivar 'Bridal Pink'. They also indicated that a combination of IAA at 0.0-0.1 mg/l and NAA at 0.0-0.1 mg/l was quite effective in induction of roots. Arnold *et al.* (1995) reported that micro shoots of *Rosa kordesii* cultivar 'John Franklin' and 'Champlain' rooted well in MS media with low or no auxin.

In the present work, Table 2.5 showed the multiplication of shoots on solid and in liquid media. Results showed that liquid MS media supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA gave higher shoot formation with 18.5 ± 0.8 shoots per explant compared to solid media with only 11.6 ± 0.5 shoots per explant. However, shoots produced in liquid media were abnormal and smaller in size. The effect of optimum sucrose concentration in culture media was also identified. Thirty g/l sucrose in culture media gave the highest shoot regeneration with 92.8 ± 0.4% while the most suitable pH of culture media ranging from 5.6-6.0 produced high regeneration of shoots. Media with pH 5.8 showed the highest shoot formation with 92.8 ± 0.5% and 9.3 ± 0.6 shoots per explant. Although 30 g/l sucrose concentration was found to be common in micropropagation of many plant species, however, it is important to study the effect of sucrose concentration in the current work since sucrose play an important role as energy and carbon source to the explants and as different species need different concentrations of sucrose. Some plant species may require higher or lower sucrose concentration for their optimum growth. This depends on the ability and requirement of the plant species. pH 5.8 was also found to be common in most tissue culture studies, however, it is important to pin point the suitable pH condition for *Gerbera* explants since it is necessary to attain almost similar pH to normal planting soil for optimal plant growth. Some soil or planting substrates require low pH (acidic) while some others may need more alkaline condition. Thus, in regeneration of *Gerbera*, it was found that pH 5.8 was the optimum pH for normal *Gerbera* growth substrate. Supplementation of coconut water in the culture media exhibited higher shoot regeneration. However, shoots produced were bushy and

smaller in size compared to shoots produced with culture media without the addition of coconut water.

The addition of sucrose at 50 g/l and 60 g/l in culture media produced vigorous *in vitro* shoots multiplication of *Amygdalus communis* L. and pH 5.5 was the optimum pH level for shoot growth of the same species (Gürel and Gülşen, 1998). The requirement of high sucrose level in the initial development and growth process may be mainly due to the fact that high sugar levels in the culture media may speed up cell division thus leading to an increase in the volume and weight of cultured tissues (Chong and Taper, 1972). However, during the proliferation and transplantation stages, the researchers discovered that lower sucrose concentration (30 g/l and 40 g/l) were significantly better during the multiplication stage in terms of shoot production and subsequent growth. Hisajima (1982a,b), Rugini and Verma (1983) and Rugini (1984b) suggested that 30g/l sucrose should be used in all culture stages for the *in vitro* multiplication of almond. This is in agreement with the present work.

Chu *et al.* (1993) reported that the growth of miniature Roses in liquid medium was better as compared to those cultured in two-phase (solid-liquid) medium or solid medium alone. The quantity of liquid medium was found to play an important role in shoot growth and multiplication of *Rosa damascene* and *Rosa bourboniana* and better shoot quality were facilitated in terms of shoot length and thickness as compared to solid medium (Pati *et al.*, 2006). Twenty ml of liquid medium was found to be the most suitable with respect to optimal shoot proliferation. Similar response was observed in

miniature Rose (Chu *et al.*, 1993), bamboo (Godbole *et al.*, 2002) and tea (Sandal *et al.*, 2001). In the present study, liquid MS media supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA produced shoot formation. However, the shoots formed were abnormal and smaller in size. The results obtained are in contrast with results reported by Chu *et al.* (1993) where solid media was suitable for formation of adventitious shoots.

Baskaran and Jayabalan (2005) reported that an efficient micropropagation system was successfully achieved for a valuable medicinal herb, *Eclipta alba* (Asteraceae) when cotyledonary node segments were cultured on MS medium with the combination of 1.0 mg/l BAP, 1.0 mg/l kinetin, 1.0mg/l 2iP, 0.5 mg/l GA_3, 5.0% coconut water and 3.0% sucrose. The medium formulation promoted maximum number of shoots as well as beneficial shoot length. *In vitro* growth and development of *Eclipta alba* was highly influenced by the concentrations of growth regulators and coconut water added to the medium. Several reports have confirmed the beneficial effect of coconut water for micropropagation of *Elettaria cardamomum* Maton (Reghunath and Bajaj, 1992); Bajaj *et al.*, 1993), pink ginger (Brain and Richard, 1993), *Amamum subulatum* Roxb. (Sajina *et al.*, 1997) and *Amomum krevanh* Pierre ex. Gagnep (Wondyifraw and Surawit, 2004). Rooting of *Eclipta alba* was highest (94.3%) on full strength MS medium supplemented with 2.0 mg/l IBA. The supplementation of coconut water in the culture media in the present study also helped in the formation of shoots from petiole explants. Media added with 100 ml/l coconut water produced 14.1 ± 1.4 shoots per explant.

Several reports have revealed the positive effects of coconut water for *in vitro* multiplication for example in *Elettaria cardamomum* Maton (Nadganda *et al.*, 1983; Bajaj *et al.*, 1993) and turmeric (Shirgurkar *et al.*, 2003). In contrast, it has been reported that coconut water was used to suppress shoot bud differentiation in *Begonia* (Heide, 1969) and *Duboisia myoporoides* (Kukreja and Mathur, 1985). Micropropagation of *Olea europaea* L. (olive) was successfully achieved when single node explants were cultured on OM (olive medium) (Rugini, 1984b)) media supplemented with 50 ml/l coconut water and 0.5 mg/l BAP (Peixe *et al.*, 2007). Coconut water is known as a natural substance with high levels of cytokinin in its composition and has high importance in micropropagation protocols of economically important species such as passion fruit (Hall *et al.*, 2000), coffee (Ismail et al, 2003) and orchids (Santoz-Hernandez *et al.*, 2005).

Microscopic studies of the structures of *in vivo* and *in vitro* leaves were done using scanning electrone microscope (SEM). It was observed that numbers of stoma were larger on abaxial leaf surfaces on both *in vitro* and *in vivo* leaves. It is known that stomata do not function properly in tissue cultured plants (Ziv *et al.*, 1987a). Normally, open stomata in tissue cultured plants cause the most significant water stress during the first few hours of acclimatization.

In tissue cultured plants, poor vascular connections between the shoots and roots may reduce water conduction. Sallanon (1993) reported that stomata of leaves from *in vitro* grown Rose plantlets remain opened in the dark. The peculiarity of guard cell ultrastructure of *in vitro* cultured plants was the inability to close in the dark. This

indicated that stomata from *in vitro* plants are duly developed and possess an ultrastructure unsuitable for a typical functioning. The inability to close in the dark results from typical water relation. Failure of stomata to close in response to darkness or externally applied ABA or high level of CO_2 was reported in apple and cauliflower propagated *in vitro* (Brainerd and Fuchigami, 1982). Environmental changes in the culture container such as a decrease in the humidity and increase of the irradiance reduced water losses (Maene and Debergh, 1987) and induced normal leaf and stomata development (Cappelades *et al.*, 1990). The cause of failure of stomata to close could be due to the guard cell wall (Ziv *et al.*, 1987a) and also the result from the stomata deformation (Blanke and Belcher, 1989).

The ultrastucture of guard cells of *in vitro* and acclimatized Rose plants is similar to many other plant species (Louguet *et al.*, 1990; Willmer, 1983). To be functional, stomata ontogenesis must occur under a not too high relative humidity and these conditions only occur in a greenhouse. Thus, it is important for all plants produced *in vitro* to be acclimatized. Acclimatization of *Gerbera in vitro* plantlets will further be discussed later.

In Chapter 3 callus induction of *G. jamesonii* Bolus ex. Hook f. was carried out in order to identify the suitable hormone concentration for optimum callus growth. The contents of secondary metabolites in *Gerbera* callus were also tested. Furthermore, the study of callus induction was also to identify the formation of embryogenic and non-embryogenic callus for the studies of somatic embryogenesis. Callus induction from leaf

and petiole explants have been successfully achieved in *G. jamesonii* Bolus ex. Hook f. with the highest fresh weight percentage of 78.8 ± 0.8% when leaf explant was cultured on MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l 2, 4-D (Table 3.1). Induction of callus from petiole explant showed the highest fresh weight percentage 70.3 ± 0.5% when explant was cultured on MS medium supplemented with the same hormone combination which was 1.0 mg/l BAP and 2.0 mg/l 2, 4-D (Table 3.2). Screening for secondary metabolites in callus of *G. jamesonii* was done using thin layer chromatography (TLC) and it was found that *Gerbera* callus extract contained flavonoid and terpenoid.

Aswath and Choudary (2002b) reported that the developmental processes of *Gerbera* callus required both cytokinins and auxins. The highest callus initiation was observed (95.0%) when leaf explant was cultured on MS medium supplemented with 4.0 mg/l NAA and 0.4 mg/l BAP. Khan *et al.* (2007) obtained efficient callus induction in *Saintpaulia ionantha* when leaves explants were cultured on MS media supplemented with 1.0 mg/l NAA and cotton was used as a support material (Khan *et al.*, 2001). Sunpui and Kanchanapoom (2002) achieved callus induction in *Saintpaulia ionantha* when petiole explants were used as explant source. Callus induction of *Kosteletzkya virginica* (Malvaceae) was highly induced (93.94%) when embryonic axes explants were cultured on MS medium supplemented with 1.0 mg/l IAA and 0.3 mg/l kinetin (Ruan *et al.*, 2009).

Abobaker *et al.* (2009) reported callus induction from radicals and cotyledon explants of *Retama raetam* (Fabaceae). Highest percentage of callus formation (41.66%)

from radicals was obtained when explants were cultured on MS medium supplemented with 5.0 mg/l NAA and 2.5 mg/l kinetin. Meanwhile, cotyledon explants induced callus (33.33%) when cultured on MS medium fortified with 4.0 mg/l NAA and 0.5 mg/l kinetin. In *Pereskia grandifolia* Haworth var. grandifolia, axillary bud explants were also the best explant for producing callus when cultured on DKW medium supplemented with 5.0 mg/l BAP and 5.0 mg/l NAA (Taha and Latif, 2007).

In some studies, 2,4-D has been shown to be most effective for callus induction in many plant species. For example Zimmerman and Read (1986) reported that picloram and 2,4-D promoted superior callus growth in *Typha* species. Rogers *et al.* (1998) observed that picloram promoted better callus growth in *Typha latifolia* when immature inflorescence explants were cultured on MS media supplemented with 1.0 mg/l and 5.0 mg/l picloram. However, picloram was not used in the present study. In *Tylophora indica* (Burm. F.), Faisal *et al.* (2005) reported that optimal callus (100%) was induced from petiole explants when cultured on MS medium supplemented with 2.2 mg/l 2,4-D and 0.5 mg/l TDZ. Meanwhile, 96% callus formation from *Dieffenbachia* cv. Camouflage (Araceae) was obtained when leaves explants were cultured on MS medium supplemented with 1.1 mg/l TDZ and 0.2 mg/l 2,4-D (Shen *et al.*, 2007). Similar growth regulators effects on callus formation have been reported in other plant species like *Duboisia* (Khanam *et al.*, 2000), *Coleus* (Reddy *et al.*, 2001), *Cassava* (Ma and Xu, 2002), *Decalepis* (Giridhar *et al.*, 2004), *Phellodendron* (Azad *et al.*, 2005), *Taxus* (Datta and Majumder, 2005) and *Ginseng* (Zhou and Brown, 2005).

Phytochemicals analysis for secondary metabolites screening done on the methanol extract of *Gerbera* callus showed that the active compounds found in the callus were flavonoid and terpenoid. Siddiqui *et al.* (2009) stated that flavonoid and terpenoid were found in many plant species. For example, flavonoid can be found in *Acacia nilotica* (Fabaceae), *Ageratum conyzoied* (Asteraceae), *Boerhaavia diffusa* (Ncytaginaceae), *Euphorbia hirta* (Euphorbiaceae), *Ficus benghalensis* (Moraceae), *Hyptis suaveolens* (Lamiaceae), *Tephrosia purpurea* (Fabaceae) and *Tridex procumbens* (Euphorbiaceae). Meanwhile, terpenoid can be found in *Cleome viscose* (Cleomaceae), *Dathura stramonium* (Solanaceae), *Prosopis juliflora* (Fabaceae), *Solanum nigrum* (Solanaceae) and *Zizyphus jujube* (Rhamaceae).

Green plants represent a reservoir of effective chemicals and provide valuable sources of natural pesticides (Balandrin and Kjocke, 1985; Hostettmann and Wolfender, 1997). Among the great variety of secondary compounds found in plants, phenolics and terpenoids represent the main antimicrobial agents known. Meanwhile, aromatic compounds such as phenol, phenolic acids, alkaloids and lectins and its derivatives such as flavonoids, tannins and coumarinns have been identified as antimicrobial agent (Siddiqui *et al.*, 2009).

In *Calendula officinalis* Linn (Asteraceae), flavonoid has been isolated from the ethanol extract of the inflorescence (Ukiya *et al.*, 2006; Kurkin and Sharova, 2007; Vidal-Ollivier, 1989) while terpenoids were extracted from the petroleum ether extract of the flower (Adler and Kasprzyk, 1975; Wilkormirski, 1985); Zittwel-Eglseer *et al.*, 1997;

Slowowski *et al.*, 1973). Al- Rehaily *et al.* (2008) reported that flavonoid and terpenoid were found in aerial parts of *Helichrysum forskahlii*. Chemical studies in *Helichrysum* species have been carried out by many researchers and the presence of flavonoids and terpenoids compounds have been reported (Bohlmann and Abraham, 1979; Bohlmann and Misra, 1984; Jakupovic *et al.*, 1989, 1990; Randriaminahy *et al.*, 1992; Caffaratti *et al.*, 1994 and Matsumoto *et al.*, 1985).

Wollenweber *et al.* (2002) reported that flavonoid and terpenoid were found in leaf extract of *Madia* species (Asteraceae). In the current study, similar result was obtained where flavonoid and terpenoid were detected in callus of *G. jamesonii*. Bohm *et al.* (1992) previously reported the occurrence of flavonoid aglycones in the leaf exudates of *Madia sativa*. In *Morinda* sp., anthraquinone is a group of natural red dye found in the root of this plant species. Aobchey et al (2002) has found an alternative way to increase the production of anthraquinone dye in *Morinda angustifolia* Roxb. by root cell culture. In *Psychotria carthagenensis* Jacq., triterpenoids were found in callus induced from leaves explants cultured on Gamborg B5 (Gamborg *et al.* 1968) medium supplemented with 0.2 mg/l kinetin and 1.0 mg/l 2,4-D (Lopes *et al.* 2000).

Callus are induced for many reasons. Besides determining secondary metabolite content in *Gerbera* callus, the present study was also aimed to induce embryogenic callus for somatic embryogenesis studies. Callus induction is the initial step towards the production of somatic embryos through indirect organogenesis. According to Narayanaswamy (1994), callus phase is important since under certain condition, it can go

through organogenesis and/or embryogenesis pathways. Unique characters of callus which is unorganized cells allow it to be induced and directed to a certain pathways. The induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control of conditions in the culture medium.

Somatic embryogenesis is a very valuable tool for achieving a wide range of objectives, from basic biochemical, physiological and morphological studies, to the development of technologies with a high degree of practical application. One of the main uses of somatic embryogenesis constitutes its employment as an approach to investigate the initial events of zygotic embryogenesis in higher plants. The mass propagation of plants through multiplication of embryogenic propagules is the most commercially attractive application of somatic embryogenesis (Merkle *et al.*, 1990).

Thus in chapter 4 somatic embryogenesis was induced from leaves explants of *Gerbera jamesonii* Bolus ex. Hook F. Indirect somatic embryogenesis was obtained in this study. Embryogenic callus was induced when leaf explants were cultured on MS medium supplemented with 0.01-2.0 mg/l 2, 4-D (Table 4.1). These callus were examined using double staining method to determine whether they were embryogenic or non-embryogenic. Observations under the microscope showed that, white-cream friable callus with early stages of embryos were formed after double staining was done, the embryonal heads stained red and suspensors stained blue. Embryogenic callus was obtained (100%) when explants were cultured on MS medium supplemented with

1.8mg/l and 2.0 mg/l 2, 4-D with the addition of 30% sucrose and 0.8% technical agar. Embryogenic callus formed were then transferred into MS cell suspension medium containing 0.1-2.0 mg/l 2, 4-D and 0.1 or 1.0 mg/l NAA for 1 month. Three weeks after the transfer of embryogenic callus to the embryo induction medium, 15.7 ± 1.4 embryos were developed in culture medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA without the addition of L-Proline (Table 4.4). Meanwhile, medium supplemented with the same concentration of growth regulators, with the addition of 50 mM L-Proline produced the highest embryo yield at 29.8 ± 1.2 embryos. Stages of somatic embryos were observed in this study from globular, heart, torpedo and cotyledonary phases.

Castillo and Smith (1997) reported direct somatic embryogenesis in *Begonia gracilis* by using micro-cultured laminar segments and petioles. The rate of somatic embryogenesis induction was greater from petiole explants compared to leaf blade explant when explants were cultured on MS medium supplemented with 0.5 mg/l kinetin and 2% (v/v) coconut water. In *Begonia x hiemalis* Fotsch (Elatior Begonia), direct somatic induction was obtained using leaf explants treated with 1.0 mg/l TIBA and cultured on MS medium supplemented with 1.0 mg/l BAP, 0.1 mg/l 2,4-D and 500 mg/l casein hydrolysate (Awal *et al.*, 2009). All cultures were incubated in the dark for 24 hours. In *Chrysanthemum*, induction of somatic embryogenesis was achieved by using leaf midrib explants (May and Trigiano, 1991). Induction of somatic embryogenesis in *Chrysanthemum* depended on the photoperiod and sucrose concentration. Somatic embryogenesis was highly produced on culture medium containing 9-18% sucrose, incubated in the dark for the first 28 days of culture, followed by 10 days in the light.

Pavingerova *et al.* (1994) reported somatic embryogenesis and plant regeneration from transformed callus of *Dendranthema grandiflora* while Tanaka *et al.* (2000) achieved the induction of somatic embryogenesis and plant regeneration in *Chrysanthemum* from ray floret explants by using IAA and kinetin. The somatic embryo derived plantlets were established in the greenhouse.

Different culture conditions were reported in inducing somatic embryogenesis in various plant species. Some plants produced somatic embryogenesis directly through the formation of embryos on the surface of explants, while some other needs to be produced indirectly through suspension culture. In the present study, somatic embryogenesis of *G. jamesonii* was obtained indirectly through suspension culture. Somatic embryos derived from leaf explants were formed (29.8 ± 1.2 per explant) in MS liquid medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA added with 50 mM L-Proline .

Direct and indirect somatic embryogenesis have also been considered as two extremes of a continuum (Williams and Maheswaran, 1986; Carman, 1990). Once induction of embryogenic cells has been achieved, they appear to be no fundamental differences between indirect and direct somatic embryogenesis (Williams and Maheswaran, 1986). Emons (1994) stated that in many systems in which embryogenesis has been designated as indirect, the embryogenic callus is composed of young embryos (pre-embryogenic masses) and that their further development depends on the duration of the application of the inductive stimulus. If the period is relatively short, the process will be direct and if it is long, the process will be indirect. On the other hand, direct

embryogenesis has been used to describe the formation of an embryo from a single cell without an intervening callus stage, although the embryo has arisen by means of the de-differentiation of a differentiated cell within the explants. Explants can be induced to form embryogenic state by a variety of procedures that usually include exposure to plant growth regulators, pH, heat shock or treatment with various chemical substances.

Somatic embryogenesis has many advantages. It permits the culture of large numbers of reproductive cells. Winkelmann *et al.* (1998) produced 90,000 plantlets from 1 litre of embryogenic cell suspension of *Cyclamen* (Primulaceae). Subsequently, Hohe *et al.* (2001) and Schwenkel (2001) reported clonal propagation of *Cyclamen persicum* by using embryogenic cell suspension culture. Pueschel *et al.* (2003) highlighted mass scale propagation of *Cyclamen persicum* via somatic embryogenesis. All the above are in agreement with the current work where mass propagation of *G. jamesonii* via somatic embryogenesis could also be obtained through suspension culture.

Saintpaulia ionantha Wendl. is commonly known as African violet is also another popular ornamental species in Malaysia. Induction of somatic embryogenesis in African violet was reported by Murch *et al.* (2003). They found that the transport of calcium and sodium play an important role in cell competence and thidiazuron (TDZ) induced somatic embryogenesis. Mithila *et al.* (2003) successfully established plant regeneration system via shoot organogenesis and somatic embryogenesis from leaf and petiole explants of greenhouse and *in vitro* grown African violet plants. Higher doses of TDZ (1.0-2.0 mg/l) were able to induce somatic embryogenesis (Mithila *et al.*, 2003). Taha *et al.* (2009)

reported that leaf explants cultured on MS medium supplemented with 1.4-5.0 mg/l TDZ formed somatic embryos on the leaf surfaces. Embryogenic callus that were transferred to MS medium supplemented with 1.8 mg/l TDZ developed further into more advanced stages and ultimately formed shoots. The results reported was in contrast to the present study whereby the supplementation of TDZ in the culture media failed to induce somatic embryogenesis in *G. jamesonii*.

Somatic embryogenesis in Rose was successful when leaf, internode, filaments of stamen, root and zygotic embryos were used as source of explants (Rout *et al.*, 1991; Roberts *et al.*, 1995, de Wit *et al.*, 1990; Kunitake *et al.*, 1993). Noriega and Sondahl (1991) and Roberts *et al.*, (1990) added ABA and GA_3 in the culture medium for the germination of somatic embryos. Li *et al.* (2002) induced somatic embryogenesis from leaf tissues of *Rosa hybrida* and *Rosa chinensis* minima. Plants were regenerated from protoplast derived embryogenic calli of *Rosa hybrida* on MS medium supplemented with 60 g/l myo-inositol, 1.0 mg/l BAP and 0.3 mg/l 2,4-D (Kim *et al.*, 2003) and the germination rate of somatic embryos was increased up to 30.9% and subsequently plantlets were established in the soil. Moghaddam and Taha (2005) reported that embryogenic callus of sugar beet had to be transferred to MS medium containing 50 mM L-Proline, 4.44 µM BAP, 1.0 µM TIBA, 3.0% phytagel and 0.35% phytagel to further develop into somatic embryos. In the present study also it was discovered that the addition of 50 mM L-Proline in the somatic embryos induction media enhanced the development of somatic embryos of *G. jamesonii*. By adding L-Proline in the primary

culture medium, embryo development was stimulated and abnormalities were reduced (Rout *et al.*, 1991).

Another advantage of somatic embryos is their utility as propagules for synthetic seeds. Somatic embryos are suitable and attractive starting materials for the production of synthetic seeds. Besides somatic embryos, other explants such as micro shoots could also be used as source of explants for the production of synthetic seed. Synthetic seeds or artificial seeds have been defined as somatic embryos engineered for use in the commercial propagation of plants (Gray and Purohit, 1991; Redenbaugh, 1993). Various forms of synthetic seeds have been envisioned over time. The first synthetic seeds formed were hydrated somatic embryos produced from vegetative cells in plant tissue culture. This method had particular advantage of enabling rapid clonal multiplication of plants. However, the labour and cost was high and the propagules were very delicate. These hydrated encapsulated embryos could only be stored under low temperatures for a few weeks (Redenbaugh *et al.*, 1986; Fujii *et al.*, 1989, 1992). The capability of prolonged storage was achieved when the somatic embryos could be dried to contain moisture contents less than 20% (McKersie *et al.*, 1989).

Several researches have been done previously on the production on synthetic seeds. For example, production of synthetic seed of *Paulownia elongata* (Ipekci and Gozukirmizi, 2003), *Saintpaulia ionantha* (Renfroe and Bolick, 1998; Taha *et al.*, 2009), Asparagus (Ghosh and Sen, 1994; Mamiya and Sakamoto, 2001), *Camellia* sp. (Janeiro *et*

al., 1997), Mulberries (Pattnaik and Chand, 2000), *Citrus reticulata* Blanco (Antonietta *et al.*, 1999) and many others.

To this date, there is no other work reporting on the production of synthetic seed of *G. jamesonii* Bolus ex. Hook f. from encapsulation of somatic embryos or other explants. The objective of experiments in chapter 5 was to establish the suitable encapsulation technique for the production of synthetic seed. Formation of idyllic, optimal, firm, uniform and round shaped beads were attempted. Several factors that affected the production of synthetic seeds were investigated. The factors include the concentration of sodium alginate ($NaC_6H_7O_6$) used as encapsulation matrix, concentrations of calcium chloride dehydrate ($CaCl_2.2H_2O$) solution as complexing agent for hardening of synthetic seeds and preparation of encapsulation matrix in different solutions. The effect of different types of sowing media on the germination of synthetic seeds was also investigated. Synthetic seeds formed were also preserved under low temperature ($4 \pm 1\ ^oC$) and the effect of storage of the seeds under low temperature was observed.

Several encapsulation techniques for the production of synthetic seeds were introduced by previous researchers such as separation funnel technique that was introduced by Redenbaugh *et al.* (1991) and mixture technique (propagules were mixed with the gelling agent). Through mixture technique, various apparatus were used to form capsule that is consists of only one propagule such as encapsulation by using forcep

(Kinoshita and Saito, 1990; Bapat *et al.*, 1987; Bapat and Rao, 1988; Ghosh and Sen, 1994).

Dupuis *et al.* (1994) introduced the development of synthetic seeds production using pharmaceutical capsules technique. Later, the double layers or hollow beads techniques based upon production of capsules with a gelled external layer surrounding a liquid internal layer was introduced (Patel *et al.*, 2000). Encapsulation technique was also done by using Pasteur pipette (Janeiro *et al.*, 1997; Ipekci and Gozukirmizi, 2003) and micro pipette (Lynch, 2002). Bapat and Rao (1988) reported that encapsulation technique done by using forcep resulted in the formation of oblong beads. According to Lynch (2002), the usage of micro pipette to form synthetic seeds managed to form firm, isodiametric and round shaped beads. Thus, micro pipette was used throughout the production of synthetic seed of *Gerbera*.

Preliminary experiment was done using micro shoots as propagules for encapsulation process. Synthetic seeds were produced from the encapsulation of micro shoots of *G. jamesonii*. Encapsulated micro shoots has the ability to break the encapsulation matrix and germinate, thus grow and develop as a complete plant. Besides somatic embryo, micro shoots are also ideal propagules for the production of synthetic seeds. Sharma *et al.* (1994) reported that micro shoot was chosen as source of propagule for synthetic seed production in *Zingiber officinale*. Other vegetative parts can also be used as propagules for encapsulation of synthetic seeds. For example, the utilisation of

apical buds for the encapsulation of *Musa* spp. (Ganapathi *et al.*, 1992) and *Valeriana wallichii* (Mathur *et al.*, 1989).

Several types of gelling agents could be used for the production of synthetic seeds such as agar, alginate, carboxy methyl cellulose, carrageenan, gelrite, guargum, sodium pectate, tragacanth gum and many others (Saiprasad, 2001). However, alginate was found to be more suitable and practicable for synthetic seed production. Alginate is normally used as gelling agent since it has special characters which are moderate viscosity, low toxicity to propagules, low cost and has bio-compatibility characteristics. The use of agar as gelling agent is deliberately avoided as it is considered inferior to alginate with respect to long term storage.

Sodium alginate is highly preferred because it enhances capsule formation and also the rigidity of alginate beads provides better protection to the propagules against mechanical injury (Redenbaugh *et al.*, 1986; Bapat *et al.*, 1987; Onishi *et al.*, 1992). According to Bapat and Rao (1990), sodium alginate matrix has complete nutrient sources and enhanced the growth of propagules at the same time helped to protect the propagules from microorganism infections. Sodium alginate also helped to prevent dehydration when the synthetic seeds were transferred to soil.

To complete encapsulation technique, calcium chloride dehydrate ($CaCl_2.2H_20$) solution was used as complexing agent. The suitable concentrations of calcium chloride dehydrate solution and seed immersion time determines the ideal synthetic seed produced. Many researchers used calcium chloride dehydrate solution as complexing

agent. Only a few had used calcium nitrate ($CaNO_3$) as complexing agent (Renfroe and Bolick, 1998).

In chapter 5, different concentrations of sodium alginate and calcium chloride dehydrate solution were used to identify the optimum concentration of these solutions to form idyllic beads. From the experiments, it was observed that sodium alginate at 3.0 % and 4.0 % formed optimal, firm, uniform and round shaped beads when the beads were left to be hardened in 100 mM (Figure 5.2) and 125 mM calcium chloride dehydrate solution for 30 minutes (Table 5.1). However, synthetic seeds produced using 3.0 % sodium alginate and hardened in 100 mM calcium chloride dehydrate solution showed optimum germination rate compared to other concentrations with 72.1 ± 0.8% (Table 5.2) when germinated on MS solid media and 69.5 ± 1.2% on MS liquid media. Meanwhile, the survival rate was 73.6 ± 1.0% on MS solid media and 70.0 ± 1.0% on MS liquid media.

Based on the experiments, it can be suggested that the production of idyllic synthetic seeds depend on the concentration of sodium alginate used as gelling agent and $CaCl_2.2H_20$ solution. The concentrations of both solutions were very important in determining the shape and rigidity of the capsules formed (Redenbaugh et al, 1991). In sodium alginate solution, the single and double ion bonds in the micronutrients and macronutrients could be the factors that initiated 'pre complexion' to occur (Barbotin *et al.*, 1993).

Taha *et al.* (2009) reported that synthetic seed of *Saintpaulia ionantha* was produced when somatic embryos were encapsulated using 3.0% sodium alginate composed in MS medium supplemented with 1.0 mg/l IAA and 2.0 mg/l zeatin. The optimum concentration of calcium chloride dehydrate used for hardening of beads was identified at 125 mM. Janeiro *et al.* (1997) reported that 3.0% of sodium alginate was most effective as gelling agent and 100 mM $CaCl_2.2H_20$ was most ideal as complexing agent. Capsules formed with 2% sodium alginate were too soft and capsules formed with 4% alginate were too hard and hindered the emergence of root and shoot. Similar observation was obtained in the present work whereby 3.0 % sodium alginate was found to be most suitable to use in the encapsulation matrix and 100 mM $CaCl_2.2H_20$ was the most suitable as complexing agent.

For the production of synthetic seeds, the encapsulation matrix was constructed when sodium alginate powder was dissolved in Ca-free MS basal liquid medium with the addition of sucrose that acts as carbon source. In this experiment, encapsulation matrix was formulated when sodium alginate powder was dissolved in 4 different solutions. Germination and survival rates of *Gerbera* synthetic seeds were observed. Encapsulation matrix composed of Ca-free MS with the addition of 2.0 mg/l BAP and 0.5 mg/l NAA showed the highest germination rate of 74.5 ± 2.6% and 75.0 ± 0.5% of survival rate (Table 5.3).

Three types of sowing media or substrates were used as germinating media, MS basal solid medium, sterile garden soil and vermiculite. Germination was 100 ± 0.0%

when synthetic seeds of *Gerbera* were germinated on MS basal solid medium (Table 5.4). The results showed that all three types of sowing media were suitable to be used as germinating media. However, MS basal solid medium is highly preferred since it produced the highest germination rate in this study. Results obtained are contrary to the results reported by Bapat *et al.* (1987) where soil was not suitable in germination of synthetic seeds of *Morus indica* L. Soil was also not suitable for the synthetic seeds germination of *Santalum album* (Bapat and Rao, 1988). Mathur *et al.* (1989) observed that *in vivo* germination of synthetic seeds of *Valeriana wallichii* on garden soil and vermiculite had caused microorganism infections and contamination of the seeds. However, intensive care has proved that *in vivo* germination of synthetic seeds of *G. jamesonii* on vermiculite and sterile garden soil was a success. The sowing media (sterile garden soil and vermiculite) were watered consistently to avoid dehydration of the synthetic seed.

Synthetic seeds of *G. jamesonii* preserved at 4 ± 1 ^{o}C and sotred under different storage periods were germinated on MS basal solid medium. It was observed that the longer the seeds were stored, the germination rates were also reduced. Synthetic seeds incubated for 30 days gave germinating rate of 91.7 ± 0.6% (Table 5.5). Meanwhile, seeds stored for 60, 90 and 120 days produced 91.0 ± 0.8%, 77.0 ± 1.0% and 53.3 ± 1.2% of germination rates respectively. The lowest germination rate was observed when the seeds were preserved for 180 days with only 14.3 ± 0.7% germination. Synthetic seeds of *Gerbera* were able to germinate when preserved at low temperatures and certain storage periods but with lower germination rates as the storage period increased.

Chapter 6 deals with regeneration of *G. jamesonii* Bolus ex. Hook f. through germination of synthetic seeds. Several factors were examined in obtaining optimum germination of *Gerbera* synthetic seeds. Several factors studied included the effects of germination media on germination period, germination and survival rates *in vitro* and *in vivo*, different sucrose concentrations in encapsulation matrix, different compositions of encapsulation matrix and the effect of storage at low temperature.

Synthetic seeds of *G. jamesonii* were constructed from the encapsulation of micro shoots (Figure 6.1) and somatic embryos (globular and cotyledonary phase) (Figure 6.2). All 3 types of synthetic seeds showed various germination and survival rates on each factor studied. Experiments were compared with the regeneration potentials of unencapsulated explants. Complete germination of synthetic seeds to form shoot and root from each propagule used were also examined.

From the experiments, synthetic seeds derived from the encapsulation of micro shoots (Figure 6.1) took 5 days to germinate on MS media supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA, while encapsulated globular and cotyledonary phase (Figure 6.2) of somatic embryos germinated in 15 and 9 days respectively (Table 6.1). Synthetic seeds formed from encapsulation of micro shoots germinated completely with the formation of shoot and root. However, incomplete germination was observed when globular and cotyledonary phases of somatic embryo were used as propagules to form synthetic seeds. Unencapsulated micro shoots took 7 days to germinate and unencapsulated globular and cotyledonary somatic embryos took 19 and 12 days

respectively. On MS solid media, encapsulated micro shoots germinated on day 6 while encapsulated globular and cotyledonary phases germinated on day 17 and 12. On MS liquid media, encapsulated micro shoots germinated on day 6, globular and cotyledonary phase somatic embryos geminated on day 18 and 12, respectively. All unencapsulated propagules were observed to germinate slightly later then encapsulated propagules. The results showed that encapsulated micro shoot germinated earlier and followed by cotyledonary phase somatic embryo and globular phase somatic embryo.

Results obtained were in contrast with the statements made by Redenbaugh *et al.* (1993), where they found that respiration of embryos in the capsules could be hindered and this reduced the germination of capsules. The type of propagules used as sources of explants could be the reason why variation and dissimilarities in germination period and rates were observed. Embryos still have the ability to undergo respiration in the capsule and further germinate. The encapsulation matrix provides nutrients to the encapsulated propagules to later germinate and develop into plantlets (Bapat and Rao, 1992; Nieves *et al.*, 1998).

Various germination rates were observed when synthetic seeds of *G. jamesonii* produced from micro shoots and somatic embryos (globular and cotyledonary phases) were left to be germinated on 5 different sowing media. Encapsulated micro shoots produced 100.00 ± 0.0% germination when germinated on MS solid media and 96.5 ± 0.6% on MS liquid media (Table 6.2). Encapsulated globular and cotyledonary somatic

embryos both showed the highest germination rate when germinated on MS solid media with 78.4 ± 0.8% and 90.6 ± 0.7%, respectively.

From the results obtained in the present work, it was found that most of the germinated synthetic seeds survived after 30 days of germination period. On MS media, encapsulated micro shoots survived with 100 ± 0.0%, encapsulated globular somatic embryo with 83.5 ± 1.0% survival rate and encapsulated cotyledonary somatic embryos with 88.4 ± 0.8% (Table 6.3). These results are the optimum survival rates reported when all encapsulated explants were germinated on MS media. All encapsulated and unencapsulated explants showed no response when germinated *in vivo* (Table 6.3). Dehydration occurred when the synthetic seeds were placed on to non-sterile vermiculite and garden soil. From the experiment, it is obvious that germination of synthetic seeds *in vivo* is not suitable. However, germination of *Gerbera* synthetic seeds could be germinated on sterile vermiculite and garden soil. Yet, it is still important to germinate synthetic seeds on non-sterile garden soil or vermiculite in order to upgrade the commercial values and the survival of germination in the field. Thus, improvements need to be made in order to allow germination of synthetic seeds on non-sterile garden soil or vermiculite.

Generally, from the results obtained, encapsulated micro shoots gave the best germination rate when compared to encapsulated globular and cotyledonary somatic embryos. Table 6.4 showed the response of germination of synthetic seeds of *G. jamesonii* (encapsulated micro shoots, encapsulated globular and cotyledonary

somatic embryos) when different concentrations of sucrose were added in the encapsulation matrix. It was observed that synthetic seeds produced with lower sucrose concentration in the encapsulation matrix were more transparent. As the concentration of sucrose in the encapsulation matrix increased, the synthetic seeds were less transparent.

Germination rates of the synthetic seeds of *G. jamesonii* were reduced with the increase of sucrose concentration in the encapsulation matrix after 8 weeks. Encapsulated micro shoots with no addition of sucrose yielded 37.0 ± 1.4% germination and 1.5 ± 0.7 shoots per explant (Table 6.4). Encapsulated globular stage somatic embryos gave 8.6 ± 0.2% germination with only 0.5 ± 1.5 shoots per explant while encapsulated cotyledonary stage of somatic embryos showed slightly higher germination (18.7 ± 0.5%) with 1.2 ± 0.2 shoots per explant. Optimum germination was observed when 30 g/l sucrose was used. Encapsulated micro shoots yielded 75.0 ± 0.5% germination with 7.5 ± 1.5 shoots per explant (Table 6.4). Encapsulated globular and cotyledonary stages somatic embryos showed 61.3 ± 1.5% with 5.6 ± 0.7 shoots per explant and 63.2 ± 1.0% with 7.3 ± 0.6 shoots per explant. It was observed that the higher sucrose concentration in the encapsulation matrix, the lower germination rates obtained. This may be due to the exceeded amount of sucrose (carbon and energy source) that is needed by the synthetic seeds to germinate. Therefore, the ability of the synthetic seeds to germinate was suppressed by the large amount of sucrose in the encapsulation matrix. In encapsulated micro shoots, germination rate of 56.3 ± 1.0% (sucrose 40 g/l) reduced to 39.5 ± 0.8% (sucrose at 60 g/l). The reduction of germination rates were also observed in encapsulated globular and cotyledonary phase somatic embryos. The results showed that germination

rates were reduced as the concentration of sucrose increased from 40 g/l to 60 g/l in the present work.

The results implied that germination of *Gerbera* synthetic seeds was affected by the sucrose concentration added in the encapsulation matrix. As widely known, sucrose is essential as source of carbon and energy to the encapsulated propagules. It is important to determine the suitable amount of sucrose that is needed for the optimum germination of synthetic seeds. The present result are supported by various reports such as in *Eucalyptus grandis* (Watt *et al.*, 2000), *Camellia reticulata* (Ballester *et al.*, 1997), *Morus indica* (Pattnaik *et al.*, 1995) and *Plumbago zeylanica* (Rout *et al.*, 2001).

Usually, encapsulation matrix was prepared by dissolving sodium alginate in Ca-free MS basal medium with the addition of sucrose. In this experiment, 3.0% sodium alginate was added in 4 different solutions to form the encapsulation matrix. Table 6.5 showed the response of germination from synthetic seeds that were composed of various types of encapsulation matrix.

Encapsulation matrix composed of MS media showed 70.5 ± 1.2% germination rate when micro shoots were used as explant with 4.0 ± 1.3 shoots per explant. Globular somatic embryos yielded 28.7 ± 1.5% germination with 2.1 ± 1.1 shoots per explant, while cotyledonary somatic embryos gave 48.8 ± 0.7% germination with 4.1 ± 1.0 shoots per explant (Table 6.5). The optimum germination (74.5 ± 2.6%) from encapsulated micro shoots were observed when encapsulation matrix used were composed of Ca-free

MS + 3 % sucrose with the addition of 2.0 mg/L BAP and 0.5 mg/L NAA with 4.6 ± 0.8 shoots per explant (Figure 6.3). Germination rate of 30.2 ± 1.2% from encapsulated micro shoots was observed when distilled water with the addition of 2.0 mg/L BAP and 0.5 mg/L NAA was used as the encapsulation matrix with 2.3 ± 0.7 shoots per explant. Encapsulated globular somatic embryos yielded 17.5 ± 2.0% of germination and 1.2 ± 0.4 shoots per explant, while cotyledonary somatic embryos gave 26.4 ± 0.8% germination with 1.8 ± 0.7 shoots per explant when the same composition was used as encapsulation matrix.

Overall, the absence of $CaCl_2.2H_2O$ in MS media as encapsulation matrix helped to increase germination potentials of the synthetic seeds. It was also observed that the addition of growth hormones (BAP and NAA) in the encapsulation matrix also aided in early germination of synthetic seeds. Moreover encapsulation matrix composed of Ca-free MS formed firm, uniform and round shaped beads. Encapsulation matrix composed of MS media and distilled water formed soft, less uniformed in size of beads.

Most of the germinated synthetic seeds (formed from various types of encapsulation matrix) survived after 8 weeks of germination. The highest survival rate was observed when Ca-free MS media supplemented with 3.0% sucrose and 2.0 mg/L BAP and 0.5 mg/L NAA were used as encapsulation matrix. Encapsulated micro shoots gave 75.0 ± 0.5% survival rate with the increase number of shoots per explant (7.5 ± 1.5) (Figure 6.4). Survival rates for encapsulated globular and cotyledonary

(Figure 5.5) somatic embryos were 61.3 ± 1.5% and 63.2 ± 1.0%, respectively (Table 6.6).

Encapsulation matrix composed of distilled water supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA showed the lowest survival rates for encapsulated micro shoots (42.5 ± 1.2%), globular somatic embryo (22.6 ± 1.7%) and cotyledonary stage somatic embryo (38.6 ± 1.2). Synthetic seeds formed from encapsulation matrix that were composed of distilled water (absence of nutrient) did not promote the germination and survival of the seeds. Thus, distilled water is not a suitable choice to be used as encapsulation matrix. In *Paulownia elongata*, synthetic seeds were produced when somatic embryos were encapsulated with 3.0% sodium alginate prepared in MS medium and submerged for 30 minutes in 50 mM $CaCl_2.2H_2O$ for hardening process (Ipekci and Gozukirmizi, 2003). Germination rate produced was 53.3 ± 1.5% while 73.7 ± 3.6% of survival rate was reported in their work. However, in papaya, encapsulated somatic embryos prepared with 2.5% sodium alginate in half strength MS medium submerged in 2 mM $CaCl_2.2H_2O$ for 10 minutes exhibited germination frequencies in the same range as unencapsulated embryos or control. Higher or lower levels of sodium alginate reduced the conversion frequency of synthetic seeds into seedlings (Castillo *et al.*, 1998).

Redenbaugh *et al.* (1993) noted that variables related to encapsulation method, including alginate type and concentration, the medium and the method used to produce the synthetic seeds were responsible for significant variations in conversion percentages

for alfalfa, carrot and celery. Sodium alginate at a concentration between 0.5% and 5.0 % was found to produce sufficiently hard capsules while still maintaining embryo integrity.

Table 6.7 showed the response of storage period on germination rates of synthetic seeds (encapsulated micro shoots, globular and cotyledonary somatic embryos). All synthetic seeds were stored under low temperature at 4 ± 1 ^{o}C for 0, 30, 60, 90, 120, 150 and 180 days. Synthetic seeds which were not incubated (0 day) were used as control. In this experiment, synthetic seeds were germinated on MS basal media for 8 weeks. Low temperature (4 ± 1 ^{o}C) was essential for reasonable post-storage viability.

Incubation of synthetic seeds was also tested at 25 ± 1 ^{o}C, however all synthetic seeds stored at this temperature failed to germinate. Only synthetic seeds which were not stored manage to germinate at 25 ± 1 ^{o}C. After 20 days of storage, all synthetic seeds were desiccated, dehydrated and lost the viability to germinate. Janeiro *et al.* (1997) reported that storage of synthetic seeds *Camellia japonica* under room temperature had reduced the viability of seeds. The synthetic seeds could not survive after two months at room temperature. The results may be attributed to a rapid desiccation of the alginate beads.

The results from Table 6.7 showed that germination of synthetic seeds were able to occur at long term storage, however longer storage period, up to 180 days reduced the quality and quantity of germination. Synthetic seeds which were not incubated showed higher germination percentage compared to incubated seeds. Non-incubated synthetic

seeds from encapsulated micro shoots germinated at 93.3 ± 0.5% while non-incubated encapsulated globular and cotyledonary somatic embryos germinated at 49.0 ± 0.8% and 67.3 ± 0.7%, respectively (Table 6.7).

Germination rates for encapsulated micro shoots reduced from 91.7 ± 0.6% (30 days storage) to 14.3 ± 0.7% (180 days storage). Germination of encapsulated globular somatic embryo decreased from 44.0 ± 1.2% (30 days storage) to 21.7 ± 1.0% (60 days storage). Encapsulated cotyledonary somatic embryos yielded 61.7 ± 1.0% of germination when incubated at 30 days and the germination rate was reduced to 32.3 ± 0.5% after 90 days of storage. Synthetic seeds formed from encapsulated globular and cotyledonary somatic embryos failed to germinate after 120 days of storage. Throughout the experiments, it was observed that encapsulated explants which failed to germinate showed changes in the quality which caused the propagules (micro shoots, globular and cotyledonary somatic embryos) to face necrosis and turned brownish and later failed to respond.

The survival rates and the percentage of somatic embryos exhibiting either complete or incomplete germination were influenced by cold storage and encapsulation. Storage period significantly reduced the survival and plant recovery frequencies of both unencapsulated and encapsulated somatic embryos of *Camellia japonica* (Janeiro *et al.*, 1997). In general, encapsulated embryos showed better resistence to storage at 4 ± 1 ^{o}C than unencapsulated embryos. Redenbaugh *et al.* (1987) stated that even at 4 ± 1 ^{o}C, the storage life of synthetic seeds of alfalfa was quite short, and plant recovery rates of

encapsulated embryos of *E. Citriodora* (Muralidharan and Mascarenhas, 1995) and *Asparagus cooperi* (Ghosh and Sen, 1994) were reduced. The report is similar to the present work where the germination quality of synthetic seeds of *G. jamesonii* was reduced as the storage period at 4 ± 1 ^{o}C increased (Table 6.7). In contrast, encapsulated embryos of *Santalum album* retained their ability to germinate (18%) after storage at 4 ± 1 ^{o}C for 45 days (Rao and Bapat, 1993). Lulsdorf *et al.* (1993) claimed that both encapsulated and non-encapsulated somatic embryos of *Picea glauca engelmannii* complex and *Picea mariana* Mill. survived one month storage at 4 ± 1 ^{o}C with no loss of germination capacity. The decline in germination into plants of stored encapsulated embryos may be related to both oxygen deficiencies in the gel beads and due to rapid drying (Redenbaugh *et al.*, 1991). Janeiro *et al.* (1995) also found that two months at 4 ± 1 ^{o}C significantly enhanced germination and plant production of 8-week-old camellia embryo clusters.

The production of synthetic seeds of *Gerbera jamesonii* Bolus ex. Hook f. is an ideal alternative for mass propagation and preservation of this important plant species. High demand of *Gerbera* plant as cut flowers and potting plant has brought awareness and attention for this research to be carried out. Conventional *Gerbera* breeding method is usually through cuttings. However, the production of synthetic seeds of this plant species has become a stepping stone towards a new and sophisticated and more efficient propagation method of *Gerbera*.

The use of *in vitro* culture methods for the selection of variant types in ornamentals has been documented for many years especially for flower colour, plant morphology and also physiological characters. *In vitro* methods has known to shorten breeding cycles and therefore reduced the costs of the development of a new cultivar (Preil, 1986). Induced variability does not seen to be different from that known to occur spontaneously. However, mutagen treatment could increase mutant frequency severely. Although some variants such as changes in flower colour may emerge from spontaneous mutations at relatively high rates, mutation frequency of many useful traits is very low (Ibrahim *et al.*, 1998). Mutagen treatments therefore, are outstanding importance for practical breeding purposes (Preil, 1986). According to Broertjes and Van Harten (1978), many ornamental species are suitable for mutation breeding, since flower colour and other mutations can be produced without altering any other characters of the original ideotype.

Gamma rays, X-rays, visible light and UV are all electromagnetic (EM) radiation. Electromagnetic radiation, having the energy level from around 10 kilo electron to several hundred kilo electron volts, and therefore they are more penetrating than other radiation such as alpha and beta rays (Kovacs and Keresztes, 2002). Gamma rays belong to ionizing radiation and interact with atoms or molecules to produce free radicals in cells. These radicals can damage or modify important components of plant cells and have been reported to affect differentially on morphology, anatomy, biochemistry and physiology of plants depending on the irradiation level. These effects include changes in the plant cellular structure and metabolism such as dilation of thylakoid membranes,

alteration in photosynthesis, modulation of the antioxidative system and the accumulation of phenolic compounds (Kim *et al.*, 2004; Kovacs and Keresztes, 2002 and Wi *et al.*, 2005).

So far, there are few work reporting on the effects of irradiation in tissue culture system by previous researchers. Most of the plant species irradiated have very high importance and commercial values such as *Saintpaulia* (Naylor and Johnson, 1937; Sparrow *et al.*, 1960; Broertjes, 1968), *Kalanchoe* (Broertjes and Leffring, 1972), *Streptocarpus* (Broertjes, 1969) and *Begonia* (Doorenbos and Karper, 1975; Roest *et al.*, 1981), *Dianthus caryophyllus* (Johnson, 1980; Simard *et al.*, 1992), *Chrysanthemum* (Breoertjes *et al.*, 1976; De Jong and Custers, 1986; Misra *et al.*, 2003), *Helianthus annus* (Omar *et al.*, 1993) and many others.

There are not many reports documented on the irradiation effects on *Gerbera* sp. and the study of irradiation effects on *Gerbera* is very limited. Chapter 7 discussed the effects of gamma irradiation on organogenesis, callus formation and also development of *Gerbera* plantlets *in vitro*, biochemical changes during post-irradiation were also studied and changes in chlorophyll content and soluble proteins of irradiated *Gerbera* callus. Experiments were carried out to investigate the irradiation effects on organogenesis and morphogenesis of irradiated callus, shoot regeneration and morphological phenotype changes of new plantlets produced.

A significant decline was observed in the number of shoots regenerated from irradiated petiole explants compared to the control in all treatments. Numbers of shoots regenerated from irradiated petiole explant cultured on non-irradiated MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA was reduced to 6.6 ± 0.9 when explants were exposed to 20 Gray of irradiation dose (Table 7.1). Number of shoots regenerated was drastically reduced to 2.5 ± 4.6 (Table 7.1, Figure 7.1 c) when explants were exposed to 60 Gray of irradiation dose. Similar observation was obtained when irradiated explants were cultured on irradiated culture medium and non-irradiated explants cultured on irradiated medium.

However, abnormalities in the shoots formed from irradiated explants were observed when explants were exposed to Gamma irradiation at 30 Gy (Figure 7.1 a), 40 Gy (Figure 7.1 b), 50 Gy and 60 Gy (Figure 7.1 c). All non-irradiated explants cultured on irradiated culture medium showed normal shoot formation. This showed that the irradiated medium did not influence shoot formation in terms of morphological changes of the shoots formed. It only influenced the number of shoots formed due to the lack of medium quality. From the results, it was observed that irradiated explants cultured in non-irradiated medium showed morphological and irradiation response of explants in the formation of stunted and abnormal shoots.

For an effective use of mutation induction in plant breeding programmes, the basic requirement is the analysis of radiosensitivity of the explant material (Walther and sauer, 1986). Ibrahim *et al.* (1998) reported that leaves of *Rosa hybrida* subjected to

increasing doses of radiation showed a decrease in regeneration capacity, which was completely suppressed at 100 Gy. The lethal dose for 50% of the regenerating explants in irradiated explants was estimated to be 25 Gy. Laneri *et al.* (1990) stated that in a mutation breeding of *G. jamesonii*, the dose chosen should result in the highest survival of irradiated explants and that a low inhibition of the rate of production of new shoots would give the highest efficiency in recovering useful mutants.

Jerzy and Zalewska (1996) irradiated leaf explants from violet pink 'Richmond' of *Dendranthema grandiflora* with X-rays and Gamma rays at doses of 5 and 15 Gy. No undesirable mericlinal or sectorial chimeras were observed in the planting materials. This indicates that adventitious shoots regenerated from leaf explants usually arise from a single cell. In this way, the development of new plants consists of genetically homogenous tissues. Ibrahim *et al.* (1998) reported that 12 new cultivars of *Rosa hybrida* were obtained after applying 15 Gy of X-ray irradiation at dose rate of 1.92 Gy/min. Morphological abnormalities are of interest in ornamental horticulture, although some reverted to normal plants when plants were subcultured, indicating that the variations might be related to some temporary physiological disturbances (Raju *et al.*, 1986).

Similar observation was reported as section 7.3.1 on effects of gamma irradiation on regeneration of *in vitro* propagated shoots. For all treatments, gradual decline was observed based on plant height as the dose of gamma irradiation increased. Irradiated *in vitro* shoots cultured on non-irradiated medium showed effect on gamma irradiation as the morphological aspects of the plantlets showed irradiation response. As the gamma

dose increased, plantlets showed irregular and abnormal characters as compared to control treatment with 8.7 ± 0.6 cm, plantlet height was reduced to half with 4.1 ± 0.7 cm when irradiated *in vitro* shoots were cultured on non-irradiated medium and when plantlets were exposed to 60 Gy of irradiation dose (Table 7.2). Meanwhile, irradiated *in vitro* shoots cultured on irradiated medium showed drastic decline to 3.1 ± 2.0 cm when plantlets were exposed to 60 Gy of irradiation dose (Table 7.2). Non-irradiated *in vitro* shoots cultured on irradiated medium showed normal morphological growth when exposed to gamma irradiation of 10-60 Gy. Three-month-old plantlets obtained from irradiated *in vitro* shoots cultured on non-irradiated culture medium were acclimatized and transferred to the green house (Figure 7.2 a-d).

Simard *et al.* (1992) reported that complete regeneration of *Dianthus caryophyllum* was achieved when petal explants were irradiated with Gamma radiation at 20 Gy and 40 Gy. Explants from various plant species have different sensitivity level towards different irradiation doses. Apart from that, the activities of exogenous hormones in the culture media were also affected by gamma irradiation. Irradiation dose may have an effect on the effectiveness of the hormones and thus, this influenced the formation of shoots. Rao *et al.* (1976) reported that gamma irradiation may prohibit the auxin activities that may change the morphogenetic responses.

Macromorphological studies was also done on *Gerbera* plantlets obtained from regeneration of petiole explants and exposed to Gamma radiation at 20 Gy, 30 Gy and 40 Gy. These plantlets were then acclimatized and transferred to the green house.

Apart from regeneration, the effect of irradiation on callus was also studied. Fresh weight percentage of irradiated callus was determined based on the formula (page 190). A significant decline in the fresh weight of irradiated callus compared to the control was observed. In this case, growth responses of callus were strongly influenced by the radiation dose. The fresh weight of callus was reduced to 76.4 ± 2.2% compared to 89.7 ± 0.5% of control (Table 7.3) when callus tissues were exposed to 20 Gy Gamma irradiation (Figure 7.3 a). Total fresh weight of callus was further reduced to 64.3 ± 0.8% (Figure 7.3 b) and 59.4 ± 0.6% (Figure 7.3 c) when callus tissues were treated with 30 Gy and 40 Gy respectively.

With increasing dose of gamma irradiation, the colour of callus continued to darken and the tissues become brown in colour and the structure of the tissue was relatively poor in contrast to the control. At 60 Gy, the fresh weight of callus tissues was reduced by half from the control with only 40.2 ± 0.5% (Table 7.3).

Though the irradiation doses exposed to the callus tissues varies, Bajaj *et al.* (1970) reported similar results on the growth and development of *Pheasolus vulgaris* as the results obtained on irradiated callus of *G. jamesonii*. According to Bajaj *et al.* (1970), the development of callus tissues declined when callus were exposed to 10-50 Gy) and drastically reduced when exposed to higher irradiation doses (200-300 Gy) while callus growth was lethal at 400 Gy of irradiation dose. Similar results were also obtained on irradiated callus of *Zea mays* (Moustafa *et al.*, 1989), *Nicotiana tabacum* (Degani, 1975;

Degani and Pickholz, 1973; Venketswaren and Partenen, 1966), *Antirrhinum majus* (Rao *et al.*, 1976) and *Helianthus annus* (Omar *et al.*, 1993).

Rao *et al.* (1976) reported that high irradiation dose (200-300 Gy) did not stimulate callus growth of *Antirrhinum majus* and the callus failed to differentiate or form leaf primordia. Wang *et al.* (1988) stated that the capability of callus tissues to differentiate and form shoots was diminished when higher irradiation dose exposed to the callus tissue.

In the present study, similar observation was reported from irradiated callus cultured on irradiated medium. As the gamma irradiation dose increased, proliferation of callus tissues declined and total fresh weight was reduced compared to control. No significant effect was observed when irradiated callus cultured on irradiated medium compared to irradiated callus cultured on non-irradiated medium. The fresh weight of non-irradiated callus cultured on irradiated medium was not significantly different from the control. The result showed that irradiated medium did not show much difference in the fresh weight of callus. The fresh weight yielded was almost similar to the control (Table 7.3).

Physiologically, gamma irradiation has effect on the cell wall and the cell membrane. The decline of callus tissue growth is affected by the high irradiation dose exposed to the tissues. Thus, this will cause changes in the cell size or the callus tissues. Effect of irradiation dose not only prohibit water intake to the cells, it may also influence

the synthesis of endogenous hormones. The reduction of callus growth may be caused by the lesser amount of endogenous hormones (auxin) in the explant.

Physiological symptoms in a large range of plants exposed to gamma rays have been described by many researchers (Kim *et al.*, 2004, 2005; Kovacs and Keresztes, 2002; Wi *et al.*, 2005). The symptoms frequently observed in the low or high dose irradiated plants are enhancement or inhibition of germination, seedling growth and other biological responses (Kim *et al.*, 2000; Wi *et al.*, 2005). The growth of *Arabidopsis* seedlings exposed to low-dose gamma rays (1-2 Gy) was slightly increased compared to control, while the seedling growth was noticeable decreased by the high dose irradiation of 50 Gy (Wi *et al.*, 2006).

Many studies have been carried out on the sensitivity and inhibition of plant growth due to irradiation in various species (Sparrow and Woodwell, 1962; Sparrow *et al.*, 1960; Davies, 1968 and Davies, 1961). The damage caused by irradiation can be expressed at the metabolic level before they appear as growth retardation and death (Inoue *et al.*, 1975). However, previous reports on the metabolic disturbance in irradiated crops in accordance to low dose irradiation have been insufficient.

Powell and Griffithhs, (1963) stated that ionizing radiation accelerated the degradation of chlorophyll in many plant species. In the present study, gamma irradiation was found to accelerate the degradation of chlorophyll in callus cultures of *G. jamesonii*. According to Giacomelli *et al.* (1967), they found that irradiation accelerated the

degradation of chlorophyll in barley leaves. Irradiation also caused some breakdown of the chloroplastic organization so that chlorophyll is more readily degraded after irradiation treatment. They reported that the primary effect of irradiation was on the development of meristematic cells and the effect of auxin supply may be a consequence of the same. Hagen and Gunckle (1958) and Kuzin (1956) stated that the alteration in chlorophyll synthesis in irradiated *Nicotiana glauca* and *Nicotiana Langsdorfii* tissues may be due to the auxin synthesis.

The chlorophyll content in fresh callus tissue is shown in Table 7.4. It was observed that as the irradiation dose increased, degradation of chlorophyll was expressed (Figure 7.4). The total chlorophyll content in the irradiated callus tissues decreased as the dose of irradiation increased. Total chlorophyll content in non-irradiated (control) was 83.2 mg/g and the amount of chlorophyll started to drop as the irradiation dose increased. Total chlorophyll was reduced to 79.8 mg/g when callus tissues were exposed to 20 Gy of Gamma irradiation. Total chlorophyll amount gradually reduced to 65.0 mg/g when callus tissue was exposed to 50 Gy. This experiment showed that gamma irradiation has significant effect, reduction in the amount of chlorophyll in callus tissues. According to Ling *et al.* (2008), lower chlorophyll content was obtained from irradiated (0-50 Gy) plantlets of *Citrus sinensis* as compared to non-irradiated plantlets. Kim *et al.* (2004) stated that chlorophyll is virtually insensitive to low doses gamma irradiation. Gamma irradiation resulted in greater reduction in the amount of chlorophyll b as opposed to chlorophyll a in *Pisum sativum* (Strid *et al.*, 1990). Meanwhile, the reduction in chlorophyll b is due to more selective destruction of chlorophyll b precursors (Marwood

and Greenberg, 1996). Abu *et al.* (2005) stated that an increase in chlorophyll a, b and total chlorophyll was observed in *Paulownia tomentosa* plants that were exposed to gamma irradiation.

Bajaj (1970) reported that high doses of gamma irradiation caused a decline in growth and inhibition of RNA and protein synthesis in bean callus culture. Both DNA and RNA were found to be lower in irradiated barley seeds and there is no clear explanation on the effect of irradiation on protein synthesis (Constantin and Love, 1967; Bajaj, 1970 and Posner, 1965).

Soluble protein content in fresh callus is shown in Table 7.5. Protein content from each treatment was obtained from protein standard curve plotted in Figure 7.5. The result showed that soluble protein content in non-irradiated fresh callus was reduced during the 3rd day of treatment with 67.4 ± 0.8 µg/g (Table 7.5, Figure 7.6). However, soluble protein content started to increase during the 5th, 7th and 9th day of treatment. After the 9th day, soluble protein content was reported to reduce and during day 15, soluble protein content in non-irradiated callus was 54.5 ± 2.5 µg/g. It can be observed that, in the irradiated callus tissues, almost similar result was obtained whereby after the 9th day of exposure to Gamma irradiation, gradual reduction of soluble protein content was reported. Reduction of soluble protein content was observed in all treatments (exposure from 10 Gy to 60 Gy) as the incubation day increased to the 15th day of treatment. The results also showed that soluble protein content decreased as the incubation day of non-irradiated and irradiated callus tissue increased. Jordan *et al.* (1992) observed a decline in

total soluble proteins in pea plants after 3 days of UV-B irradiation whereas in other species, including a different cultivar of corn, the protein content did not change (Basiouny *et al.*, 1978).Tiburcio *et al.* (1985) reported that the soluble protein level in recovered in *Nicotiana rustica* plants compared to those of the control plant or just irradiated plants was higher indicating that stimulatory effect on the increase of soluble protein content may be due to post-treatment recovery.

In corn seeds, Cherry *et al.* (1961) noted that the production of reducing sugar, protein, soluble nucleotides and RNA was reduced by X irradiation and indicated that this reduction was parallel to growth reduction. Meanwhile, Kuzin (1956) and Hagen and Gunckle (1958) found a depression of protein synthesis in *Nicotiana glauca* and *Nicotiana langsdorfu* caused by radiation. Glasson and Lee (1971) also reported that irradiation caused a breakdown of protein in tomato fruit. Meanwhile, Vasil (1962) observed an increase in protein synthesis in a variety of plant species a few days after irradiation.

From this study also, macro morphological observation showed that, with the increase of gamma irradiation dose, smaller plantlets with small leaves sizes were obtained. Plantlets leaves were much glossy and less hairy compared to normal plantlets.

To complete the whole tissue culture process, plantlets obtained need to be acclimatized successfully to the normal environment. *Gerbera jamesonii* is well known as a temperate grown ornamental plant. Popular demand towards this plant has inspired

this research to be accomplished in Malaysia, a tropical climate country. Through plant tissue culture techniques, *G. jamesonii* plantlets were successfully regenerated.

A substantial number of micropropagated plants failed to survive the transfer from *in vitro* conditions to the green house or field environment. The green house have substantially lower relative humidity with higher light level and septic environment that are stressful to the micropropagated plants compared to *in vitro* conditions. Most species grown *in vitro* require acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil.

Smith *et al.* (1990) reported that plantlets survival will increase when transferred to a lower relative humidity environment. Thus, the low relative humidity condition of the culture vessels is important in order to develop more vigorous plantlets structure and therefore, plantlets could adapt easily when transferred to the field (Ziv *et al.*, 1983).

In chapter 8, acclimatization of *in vitro* plantlets of *G. jamesonii* to the greenhouse was carried out. Adaptation of *Gerbera* plantlets to the new environment was observed. Plantlets were transplanted to four different media, garden soil (combination of black soil and red soil at 2:1), autoclaved garden soil (combination of black soil and red soil at 2:1), vermiculite and red soil. Plantlets of *G. jamesonii* responded optimally when acclimatized in garden soil (combination of black soil and red soil at 2:1 ratio. This treatment gave the highest survival rate for acclimatization *G. jamesonii* with the

percentage of 86.0 ± 0.9%. This soil mixture is suitable for acclimatization of *G. jamesonii* since it provide good drainage system for the plant. Sink (1984) stated that retain water will deplete oxygen level in the soil and thus, this causes failure of the plant rooting system. However, plantlets acclimatized in autoclaved garden soil failed to survive after 1 week of transfer. Plantlets acclimatized in vermiculite also showed healthy growth with 73.0 ± 1.3% survival rate. Meanwhile, plantlets acclimatized in red soil hardly survived with lower survival rate at 37.5 ± 1.0%.

Various growth substrates could be used for acclimatization purposes. Reynoird *et al.* (1993) rooted *in vitro* shoots with half-strength MS containing 0.05 mg/l NAA and acclimatized regenerated plant using peat and perlite (1:1) medium and achieved 100% success. Petru and Matous (1984) successfully transferred the plantlets to sterilized peat and perlite (1:1) substrate and then to a standard horticultural substrate. Meanwhile, 95% survival was achieved when rooted *Gerbera* plantlets were transferred to Jiffy-7 peat pellets, in glass covered acclimatization module and later to a mixture of perlite and spaghnum moss (1:1) in the greenhouse (Laliberte *et al.*, 1985). Aswath and Choudhary, (2002b) reported that *Gerbera* plantlets exhibited 100% survival rate in plastic pots filled with coco peat, red soil and sand at ratio of 3:1:1 while Parthasarathy and Nagaraju (1999) observed 90-100% survival rate when *Gerbera* plantlets were acclimatized in polythene bags containing equal amount of soil, sand and manure. Ray *et al.* (2005) reported that the plantlets micropropagated in garden soil were uniform and identical to the mother plants with respect to growth characteristics and morphology.

In micropropagation of curry leaves (Hazarika *et al.*, 1995) and citrus (Hazarika *et al.*, 1999), acclimatization was successful when plantlets were transferred to soilrite with farmyard manure as a carrier. Parthasarathy *et al.* (1999) reported that very high *ex vitro* survival (90-97%) was achieved in citrus using these carriers. Meanwhile, Da Silva *et al.* (2003) found that acclimatization of *in vitro* plantlets of *Sinningia speciosa* was achieved when plantlets were acclimatized using Plantmax or the mixture of Plantmax and vermiculite.

A heterotrophic mode of nutrition and poor mechanism for water loss control further renders micropropagated plants vulnerable to transplantation shocks. Therefore, transfer of individual plantlet to a potting mixtures and their acclimatization under greenhouse conditions require the application of various methods to harden the plants for transplantation. Although different workers using different growth substrates achieved 50-100% success in acclimatization of *Gerbera*, still the technology needs improvement to achieve 100% success in all species to meet the growing demand of growers globally.

The realization of large scale micropropagation depend on the ability and success of transplantation on *in vitro* plantlets to the greenhouse and consequently show high survival rates with low material cost (Bhojwani and Dhawan, 1989; Zaid and Hudges, 1995). Acclimatization process can be done while plantlets were still in aseptic condition or in the culture media to ensure high survival once the plantlets being transplanted to the soil or other growth substrate. Early stage acclimatization has lead to a higher survival of *Gerbera* plantlets in the present study.

In vitro plantlets of *G. jamesonii* were successfully regenerated from petiole explants, somatic embryo, germination of synthetic seeds (derived from micro shoots and somatic embryo) and *in vitro* plantlets that have been irradiated with Gamma ray at 20, 30 and 40 Gy. All plantlets were transplanted successfully in best sowing medium (garden soil) (Figure 8.2, 8.3). Acclimatization process was achieved and all plantlets were able to adapt and survived under the new environment in the green house. Plantlets regenerated from petiole explant gave highest survival rate of 86.0 ± 0.9 %. Acclimatization of plantlets derived from the induction of somatic embryo resulted with 64.2 ± 0.2 % survival. Two types of acclimatization of plantlets obtained from germination of synthetic seeds were studied. Firstly, synthetic seeds derived from micro shoots and secondly, synthetic seeds derived from somatic embryos. The survival rates of acclimatization for these 2 types of plantlets did not differ very much. Acclimatized plantlets obtained from germination of synthetic seeds derived from micro shoots survived at 73.0 ± 0.4%. Meanwhile, plantlets obtained from germination of synthetic seeds derived from somatic embryos survived acclimatization process with 70.0 ± 0.1 % survival rate. Acclimatization process for *in vitro* plantlets irradiated with Gamma ray at 20 Gy, 30 G and 40 Gy were also observed. Among the three, acclimatized irradiated plantlets at 20 Gy resulted with 40.1 ± 0.1 % survival rate, followed by 15.0 ± 0.5% and 4.4 ± 0.6% for acclimatized irradiated plantlets at 30 Gy and 40Gy, respectively (Table 8.2).

In vitro plantlets of *G. jamesonii* regenerated from petiole explant, induction of somatic embryos, germination of synthetic seeds derived from micro shoots and somatic

embryos were successfully acclimatized in the greenhouse. Meanwhile, irradiated *in vitro* plantlets by exposing the plantlets to Gamma radiation at 20, 30 and 40 Gy gave different responses on acclimatization process. Plantlets irradiated at 20 and 30 Gy were able to be acclimatized. However, survival rate for plantlets irradiated at 40 Gy were very low with only 4.4 ± 0.6% of survival. All successful acclimatized plantlets showed healthy growth in the culture room and in the green house.

Chlorophyll content in intact, *in vitro* plantlets and acclimatized *in vitro* plantlets were investigated using SPAD (Soil Plant Animal Department of Minolta) meter. Intact plant showed chlorophyll reading at 53.1 ± 0.5 SPAD. Meanwhile *in vitro* plantlet showed chlorophyll reading at 32.0 ± 0.1 SPAD. Three types of acclimatized *in vitro* plantlets, 2-month-old, 5-month-old and 12-month-old acclimatized *in vitro* plantlet showed SPAD reading at 33.5 ± 0.7 SPAD, 40.7 ± 1.0 SPAD and 49.4 ± 0.2 SPAD respectively (Figure 8.4). The results showed that as the plantlets grew older and reached maturity, the chlorophyll level increased. Lee *et al.* (1985) reported that lower chlorophyll content have been demonstrated in tissue cultured plantlets of *Liquidambar styraciflua L.* compared to seedlings. Lower chlorophyll content was also observed in *in vitro* grown coconut plantlets compared to acclimatized palm (Triques *et al.*, 1997).

All acclimatized *in vitro* plantlets in the greenhouse showed almost similar leaf morphology. Through observation, acclimatized plantlets obtained from regeneration of petiole explant, induction of somatic embryo and germination of synthetic seeds showed green, rutted, thick and coarse with silky hair leaf texture. Acclimatized irradiated

plantlets showed thinner and lighter green leaf as compared to non-irradiated plantlets. Plantlets exhibited the same 'obovate' leaf shape, 'obtuse' leaf apices, 'lobed' leaf margins and 'basal rossette' leaf arrangements. These characteristics were evident in all acclimatized *in vitro* plantlets. However, plantlets leaves sizes were varied.

In vivo plantlets have 'oblong' to 'obovate' leaf shape. Other than that, *in vivo* plants also showed almost the same morphological characters as acclimatized *in vitro* plantlets. The differences are in plant height and size. Flowering was successful for *in vivo* plants and acclimatized *in vitro* plantlets (Figure 8.5, 8.6). Flower characteristics were compared between *in vivo* plants and acclimatized *in vitro* plantlets. Both plants produced flowers with similar characters. Flowers produced are pink in colour (Figure 8.7, 8.8) and with 'actinomorphic' symmetry.

Flower colour is controlled by a dominant gene for each colour pigment, while number of flowers produced is mainly under the control of many genes (Langton, 1980). This shows that each gene has no specific potential that control flower colour variation as could be observed on flower colour. The difference that could be observed depends on the genetic content of the mother plant and the sensitivity of the plant towards radiation (De Jong and Custers, 1986). Therefore, plant size, plant height, and leaf size are controlled by a gene that is dependant on plant genotype, where plant genotypes rely on the response after induction by gamma irradiation. Tubiana *et al.* (1986) reported that the destruction of nucleic content caused by irradiation is the main reason of the damage of

explant cells. The damage that occurs on the membrane and cytoplasm is not as critical compared to the damage on the nucleic content.

Based on the results from Table 8.3, gamma irradiation has changed the plant phenotype characters especially on the plant height and leaf size. Simard *et al.* (1992) reported that acclimatized *in vitro* plantlets of *Dianthus caryophyllus* produced flowers with spots and fine lines. These flower patterns will cause changes to the basic flower colour. Irradiated *in vitro* plantlets of *G. jamesonii* however failed to produce flowers when acclimatized to the green house. Physiologically this is caused by the genotype or genetic composition of the species. In nature, flowering of *Gerbera* plants will normally occur after 6 months of cultivation.

Gerbera jamesonii has gained popularity in the past few years in many countries of the world and it is in great demand in the floriculture industry as cut flowers as well as potted plant due to its beauty, colour, long vase life and ability to rehydrate after long transportation. The study of tissue culture and morphogenesis of *G. jamesonii* has widened the interest in exploring alternative method of propagating this commercial ornamental plant. Through experimental research that has been completed in this thesis such as *in vitro* micropropagation, callus induction, somatic embryogenesis, the production of synthetic seeds, effects of gamma irradiation on growth and development of *Gerbera* plantlets and acclimatization of *Gerbera* plantlets to new environment, many new information and knowledge regarding the production of *Gerbera* plants through various methods could be spread and applied to various fields such as horticulture,

floriculture, etc. It is hoped that this research would be highly beneficial to many other plant species as reference towards exploring the astonishing tissue culture field. It is important to note that tissue culture studies is the key fundamental studies towards other research that involves molecular, genetics, cytology, and many other fields in biotechnology.