CHAPTER 8

ACCLIMATIZATION OF PLANTLETS OF *Gerbera jamesonii* Bolus ex. Hook f.

8.1 EXPERIMENTAL AIMS

Acclimatization is an important step in micropropagation. It is an adaptation process to the natural environment for various plant species which has undergone growth and development process *in vitro* (Preece and Sutter, 1991). Most species grown *in vitro* require an acclimatization process in order to ensure that sufficient number of plants able to survive and grow vigorously when transferred to soil.

According to Mohamed and Vidaver (1990), in tissue culture system, acclimatized *in vitro* plantlets showed almost the same characteristics as intact plant. The benefit of any micropropagation system can only be realized by the successful transfer of plantlets from tissue culture system to the natural environment found ex-vitro.

Morphological characters could clearly be seen when *in vitro* plantlets were successfully transferred to soil through acclimatization process. A small percentage of *in vitro* plants showed morphological and cytological changes that are termed as somaclonal variation (Larkin and Scowcroft 1981; Evans, 1989). Stresses during acclimatization are one of the factors that influence variation in most plants. This shows that acclimatization of *in vitro* plantlets could not guarantee that the new plant produced through *in vitro* multiplication is true-to-type to the mother plant in genotype aspect (Swartz, 1991).

The objective of this chapter was to obtain the most suitable technique of acclimatization for *Gerbera* plantlets regenerated from tissue culture system (*in vitro*). At the same time, the effects of radiation and somaclonal variations on *Gerbera* plantlets to produce mutant plants were also investigated.

All *in vitro* plantlets obtained from earlier experiments (Chapter 2, 3, 4, 5, 6, 7 and 8) were transferred to the green house for the acclimatization process. Garden soil (combination of black and red soil) and vermiculite were used as planting media or substrates. The selection of suitable substrates or media can be a major decision for acclimatization. Macro morphological studies were done on each plant in order to detect somaclonal variation that might occur throughout culture period. Comparisons between *in vitro* and intact plants were also made. It is hoped that *G. jamesonii* plants established through tissue culture system would give rise to somaclonal variation characteristics that could have potential for commercialization.

8.2 MATERIALS AND METHODS

8.2.1 Source of *In vitro* Plantlets

Shoot regeneration from *G. jamesonii* was successfully achieved from petiole explants. The optimum shoot regeneration was obtained when the explants were cultured on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA after 8 weeks. These shoots were then transferred to MS basal medium for rooting induction. After 3 months, new plantlets obtained from *in vitro* cultures were ready to be transferred to the green house.

8.2.2 Development and Growth of *In Vitro* Plantlet

8.2.2.1 Transplantation of *In Vitro* Plantlets to Various Media or Substrates and Acclimatization Process

Three–month-old plantlets of *G. jamesonii* obtained from tissue culture system were transferred to the green house. Plantlets were removed carefully from the culture vessels and the roots were rinsed with sterile distilled water to remove excess agar from the roots. Plantlets were transferred to various growth substrates such as garden soil and vermiculite. Plantlets were planted in small pots with size 80x60 mm. The plantlets were first kept in the culture room at 25 ± 1 ^{o}C under 16 hours light and 8 hours dark for 3 weeks before being transferred to the green house. Initially, the plant pots were covered

with plastic covers with small holes to allow adaptation and adjustment process of plantlets to the new environment. All plantlets were watered everyday. After 3 weeks in the culture room, plantlets were ready for the next step of growth and transferred to the green house. Plantlets were transferred to 4 different scheme of growth medium;

1) Garden soil - Combination of black soil and red soil at 1:2 ratio
2) Garden soil - Combination of black soil and red soil at 1:2 ratio (autoclaved)
3) Vermiculite
4) Red soil

Based on all experiments done, the best acclimatization technique which gave the highest survival rates was identified.

8.2.3 Transferring of Plantlets to the Green House and Acclimatization of *Gerbera* Plantlets Obtained from Various Culture Protocols

All *in vitro* plantlets of *G. jamesonii* obtained from previous experiments were acclimatized and transferred to the green house. These plantlets were obtained from different pathways or culture protocols as below:

1) *Gerbera* plantlets obtained from regeneration of petiole explants
2) *Gerbera* plantlets obtained from somatic embryos

3) *Gerbera* plantlets obtained from germination of synthetic seeds derived from micro shoots
4) *Gerbera* plantlets obtained from germination of synthetic seeds derived from somatic embryos
5) *Gerbera* plantlets obtained from regeneration of petiole explants and exposed to Gamma radiation at 20 Gy, 30 Gy and 40 Gy

All of these plantlets were transferred and acclimatized in the green house using the best sowing media. All plantlets used were of the same age, height and leaves number and size.

8.2.4 The Effect of Different Environmental Factors on Acclimatization of *Gerbera jamesonii*

The growth of acclimatized plantlets is also greatly affected by environmental factors such as relative humidity, temperature, light intensity and photoperiod. *Gerbera jamesonii* is a native of temperate countries. In this experiment, it is important to find out whether this plant can survive at environment with lower or higher temperatures. It is best to choose light and temperature conditions that are best suited for the acclimatization process in order to achieve optimum plant survival. The hardening process of acclimatized plantlets took place at two different locations. Plantlets transferred to garden soil were placed in the culture room with higher relative humidity at 25 ± 1 ^{o}C with 16 hours light and 8 hours dark and in the green house with higher temperature at 28 ± 1 ^{o}C

with 12 hours light and 12 hours dark. The survival and growth of acclimatized plantlets were compared. Results were recorded after 9 months of acclimatization process.

8.2.5 Measurement of Cholorophyll Content

The chlorophyll content in intact plant, *in vitro* plantlets, 2-month-old, 5-month-old and 12-month-old acclimatized *in vitro* plantlets were compared using SPAD meter (Soil Plant Animal Department of Minolta).

8.2.6 Macromorphology Studies

Macromorphology studies of plantlets obtained from various sources after being transferred to the green house (section 8.2.3) were made, recorded and compared with the intact (*in vivo*) plants. Plantlets were characterized according to several features such as plant height, size and texture of leaves and also size, symmetry, height and colour of the flowers.

8.2.7 Data Analysis

Data obtained were analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at p=0.05

8.3 RESULTS

Plantlets obtained from *in vitro* cultures need to be transferred and acclimatized under the green house conditions in order to ensure the success of micropropagation. This process requires adaptation of plantlets to the new or natural environment. The success of *in vitro* propagation is reliant by the accomplishment and success of plantlets acclimatization and transplantation to the green house. Most species grown *in vitro* require an acclimatization process in order to ensure high efficiency of plant survival rate when transferred to the natural environment.

8.3.1 Transferring of *In Vitro* Plantlets to Various Media or Substrates and Acclimatization Process

Plantlets were transferred to four different media (section 8.2.2.1). The adaptation of the plantlets to the new environment was examined. Response on the survival of *G. jamesonii* was observed and showed in Table 8.1. Results were obtained after 4 weeks plantlets being acclimatized to the new growth media.

Plantlets 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 of *G. jamesonii* (Figure 8.3) with the percentage of 86.0 ± 0.9%. However, the leaves of plantlets acclimatized in autoclaved garden soil wilted after 3 days of acclimatization and failed to survive after 1 week of transplantation. Plantlets

acclimatized in vermiculite also showed healthy growth with 73.0 ± 1.3% survival rate. Meanwhile, majority of plantlets acclimatized in red soil hardly survived. Lower survival rate of 37.5 ± 1.0% was recorded.

From the observations, plantlets acclimatized in garden soil showed optimum response and healthy growth. The development of these plantlets was showed with the increase number of leaves and sizes. Plantlets were maintained in the greenhouse for further maturation and flowering process.

Table 8.1: Responses showed by *in vitro Gerbera* plantlets after being acclimatized in various sowing media. Results obtained after 4 weeks plantlets being acclimatized

Method	Observations	Survival of *Gerbera* plantlets (%)
Plantlets cultured in MS basal medium were removed carefully from the medium and the roots were rinsed with sterile distilled water. Plantlets were then acclimatized in garden soil (combination of black soil and red soil at 2:1 ratio)	Plantlets survived and showed healthy growth	$86.0 \pm 0.9_a$
Plantlets cultured in MS basal medium were removed carefully from the medium and the roots were rinsed with sterile distilled water. Plantlets were then acclimatized in autoclaved garden soil (combination of black soil and red soil at 2:1 ratio)	Plantlets failed to survive after 1 week being transferred to autoclaved soil	0.0_d
Plantlets cultured in MS basal medium were removed carefully from the medium and the roots were rinsed with sterile distilled water. Plantlets were then acclimatized in 'vermiculite'.	Plantlets survived and showed healthy growth	$73.0 \pm 1.3_b$
Plantlets cultured in MS basal medium were removed carefully from the medium and the roots were rinsed with sterile distilled water. Plantlets were then acclimatized in red soil	Plantlets survived but showed slow growth	$37.5 \pm 1.0_c$

Mean ± SE, n=30. Mean with different letters in the same column differ significantly at p=0.05

8.3.2 Transferring of Plantlets to the Green House and Acclimatization of *Gerbera* Plantlets Derived from Various Treatments

In previous experiments, *in vitro* plantlets of *G. jamesonii* were successfully regenerated from petiole explants, somatic embryos, 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 (section 7.3.3). All plantlets were transferred successfully in the best sowing medium (garden soil) (Figure 8.2, 8.3). Acclimatization process was achieved and all plantlets were able to adapt and survived with the new environment in the green house. Survival rates of these plantlets were observed and recorded (Table 8.2). Plantlets regenerated from petiole explants gave the highest survival rate of 86.0 ± 0.9%. Acclimatization of plantlets derived from the induction of somatic embryos 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 after 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 showed survival rate of 70.0 ± 0.1%.

Acclimatization process for *in vitro* plantlets irradiated with Gamma ray at 20 Gy, 30 Gy 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 40 Gy, respectively (Table 8.2).

8.3.3 The Effect of Different Environmental Conditions on Acclimatization

Environmental factors play very important roles in determining the success of acclimatization process. Plants need suitable environment with good humidity, temperature and light that will allow good development and growth process.

Gerbera jamesonii is known as a temperate grown plant. Thus, they normally need environment with cool temperature for optimum growth. In this experiment, plantlets transferred in garden soil placed in the culture room with higher relative humidity at 25 ± 1 ^{o}C with 16 hours light and 8 hours dark and in the green house with higher temperature of 28 ± 1 ^{o}C with 12 hours light and 12 hours dark both showed good responses and developed well (Figure 8.1). However, plantlets acclimatized in the culture room failed to flower after 9 months being kept in the culture room. Plantlets acclimatized in the green house successfully flowered after 6 months.

Table 8.2: Responses showed by *in vitro Gerbera* plantlets obtained from various sources after being acclimatized in garden soil. Results obtained after 12 weeks plantlets being acclimatized

Methods	Observation	Survival of *Gerbera* plantlet (%)
Plantlets obtained from regeneration of petiole explant were removed carefully from the medium and the roots were rinsed with sterile distilled water	Plantlets survived and showed healthy growth	$86.0 \pm 0.9_{a}$
Plantlets obtained from induction of somatic embryos were removed carefully from the medium and the roots were rinsed with sterile distilled water	Plantlets survived and showed healthy growth	$64.2 \pm 0.2_{b,c}$
Plantlets obtained from regeneration of synthetic seeds (synthetic seeds derived from micro shoots) were removed carefully from the medium and the roots were rinsed with sterile distilled water	Plantlets survived and showed healthy growth	$73.0 \pm 0.4_{b}$
Plantlets obtained from regeneration of synthetic seeds (synthetic seeds derived from somatic embryos) were removed carefully from the medium and the roots were rinsed with sterile distilled water	Plantlets survived and showed healthy growth	$70.0 \pm 0.1_{b}$
Plantlets obtained from regeneration of petiole explant and exposed to Gamma radiation at 20 Gy, removed carefully from the medium and the roots were rinsed with sterile distilled water	Plantlets survived but showed some morphological variation (plant height and leaves morphology)	$40.1 \pm 0.1_{c}$

'Table 8.2, Continued'

Methods	Observations	Survival of *Gerbera* plantlets (%)
Plantlets obtained from regeneration of petiole explant and exposed to Gamma radiation at 30 Gy, removed carefully from the medium and the roots were rinsed with sterile distilled water	Some plantlets died after 1 week being transferred. Survived plantlets showed some morphological variation (plant height and leaves morphology)	$15.0 \pm 0.5_d$
Plantlets obtained from regeneration of petiole explant and exposed to Gamma radiation at 40 Gy, removed carefully from the medium and the roots were rinsed with sterile distilled water	Most plantlets died after 1 week being acclimatized	$4.4 \pm 0.6_e$

Mean ± SE, n=30. Mean with different letters differ significantly at p=0.05

Figure 8.1: The effect of different environmental conditions on acclimatization of *Gerbera jamesonii*

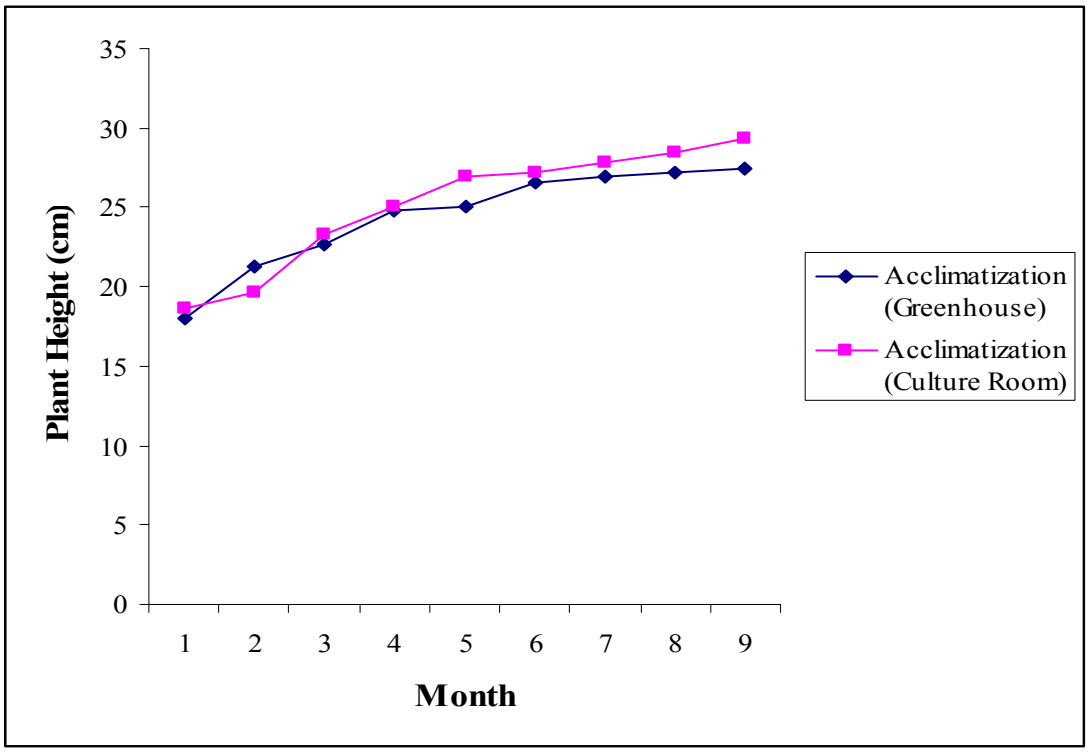


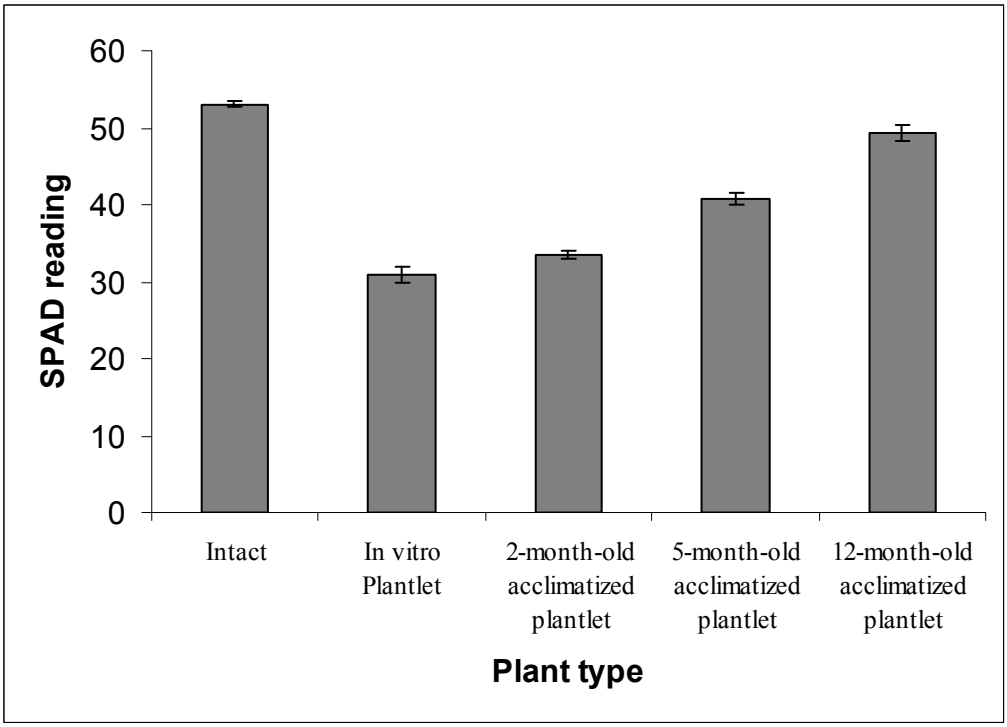
Figure 8.2: Two-month-old *in vitro* plantlets growing on garden soil and covered with plastic for acclimatization process

Figure 8.3: Three-month-old *Gerbera jamesonii* plantlets ready to being acclimatized in the green house

8.3.4 Measurement of Chlorophyll Content

Chlorophyll content is essential for the efficiency of photosynthesis process in plants. In the present work, chlorophyll content in intact, *in vitro* plantlets and acclimatized *in vitro* plantlets (obtained from plantlets regenerated from petiole explants) 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 plantlets i.e. 2-month-old, 5-month-old and 12-month-old acclimatized *in vitro* plantlets showed SPAD reading at 33.5 ± 0.7 SPAD, 40.7 ± 1.0 SPAD and 49.4 ± 0.2 SPAD, respectively (Figure 8.4).

Figure 8.4: Comparison of chlorophyll content between intact plants, *in vitro* plantlets, 2-month old, 5-month-old and 12-month-old acclimatized *in vitro* plantlets



8.3.5 Macromorphology Studies of *Gerbera jamesonii*

Previous experiments showed that *in vitro* plantlets regenerated from petiole explants, 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 (section 8.2.3) gave different responses during acclimatization process. Plantlets irradiated at 20 and 30 Gy managed to grow and were able to be acclimatized. However, survival rate for plantlets irradiated at 40 Gy was very low. All successful acclimatized plantlets showed healthy growth in the culture room and also in the green house. The development and growth for all plantlets including irradiated plantlets were observed based on morphological characters such as height of plantlets and leaf morphology. These characteristics were summarized in Table 8.3.

All acclimatized *in vitro* plantlets in the greenhouse showed almost similar leaf morphology. Through observation, acclimatized plantlets obtained from regeneration of petiole explants, induction of somatic embryos and germination of synthetic seeds showed green, rutted, thick and coarse with silky hair leaf textures. Acclimatized irradiated plantlets showed thinner and lighter green leaves 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 leaf height and size were varied (Table 8.3).

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 were in plant heights and sizes. Flowering was observed for *in vivo* plants and acclimatized *in vitro* plantlets (Figure 8.5, 8.6). However, irradiated *in vitro* plantlets were unable to flower. Thus, flower characteristics were only reported for *in vivo* plant and acclimatized *in vitro* plantlets. *In vivo* plants resulted in taller plants (24.4 ± 0.3 cm) height compared to *in vitro* plantlets (22.6 ±0.5 cm) and irradiated *in vitro* plantlets at 20 Gy (19.0 ± 1.2 cm), 30 Gy (17.8 ± 0.7 cm) and 40 Gy (17.1 ± 0.4 cm).

Flower characteristics were compared between *in vivo* plants and acclimatized *in vitro* plantlets. Both plants produced flower with similar characters. Flowers produced were pink in colour (Figure 8.7, 8.8) and with 'actinomorphic' symmetry. Characters distinguished between *in vivo* plant and *in vitro* plantlets were peduncle height, number of petals, number of flowers and flower sizes (diameter).

Based on all observations and comparison, it can be concluded that *in vivo* plants were taller in height, bigger leaf size as well as larger flower number and size as compared to acclimatized *in vitro* plantlets and irradiated plantlets.

Table 8.3: Comparison of macromorphological characters of *in vitro* plantlets, intact plants (control) and irradiated plantlets at 20 Gy, 30 Gy and 40 Gy after 6 months being acclimatized on garden soil.

Characteristics	*In vivo* Plant (control)	*In vitro* Plantlet	Irradiated Plantlet		
			20 Gy	30 Gy	40 Gy
Plant Height (cm)	24.4 ± 0.3	22.6 ±0.5	19.0 ± 1.2	17.8 ± 0.7	17.1 ± 0.4
Leaf morphology					
➢ Texture	Rutted, thick, coarse with silky leaf hair	Rutted, thick, coarse with silky leaf hair	Rutted, less thick, coarse with fine silky leaf hair	Rutted, less thick, coarse with fine silky leaf hair	Rutted, less thick, coarse with fine silky leaf hair
➢ Form	Simple, lobed	Simple, lobed	Simple, lobed	Simple, lobed	Simple, lobed
➢ Colour	Green	Green	Lighter green	Lighter green	Lighter green
➢ Shape	Oblong to Obovate	Obovate	Obovate	Obovate	Obovate
➢ Apices	Obtuse	Obtuse	Obtuse	Obtuse	Obtuse
➢ Margin	Lobed	Lobed	Lobed	Lobed	Lobed
➢ Arrangements	Basal 'rossette'	Basal 'rossette'	Basal 'rossette'	Basal 'rossette'	Basal 'rossette'
➢ Size (3rd leaf from new shoot)					
- length (cm)	21.3 ± 0.2	19.3 ± 0.6	15.4 ± 0.7	12.9 ± 0.5	12.0 ± 1.0
- width (cm)	8.2 ± 0.5	7.1 ± 0.5	4.6 ± 1.0	3.7 ± 1.3	3.3 ± 0.4
Flower characteristics					
➢ Peduncle length (cm)	29.7 ± 0.5	28.6 ± 0.2			
➢ Symmetry	Actinomorphic	Actinomorphic			
➢ No. of flower/ plant	2.8 ± 0.1	2.2 ± 0.3	Non-flowering	Non-flowering	Non-flowering
➢ Colour	Pink	Pink			
➢ Flower size (diameter, cm)	8.2 ± 0.4	7.6 ± 0.1			
➢ Flower scent	None	None			

Figure 8.5: Flower bud produced after 23 weeks *in vitro* plantlet being acclimatized in the green house

Figure 8.6: Six-month-old *Gerbera* plantlet obtained from *in vitro* propagation acclimatized in the green house with flower bud

Figure 8.7: Six-month-old flowering *Gerbera* plant obtained from regeneration of petiole explant

Figure 8.8: Flower of *Gerbera jamesonii* from *in vitro* plantlet after being acclimatized for 6 months in the green house.

8.4 SUMMARY OF RESULTS

1. *In vitro* plantlets of *G. jamesonii* Bolus ex Hook f. obtained from various experiments were successfully acclimatized.

2. Garden soil (combination of black soil:red soil at 2:1 ratio) was identified as the most suitable sowing medium for acclimatization of *in vitro Gerbera* plantlets. Plantlets cultured in MS basal medium were removed carefully from the medium and the roots were rinsed with sterile distilled water. Plantlets were then acclimatized in garden soil.

3. Plantlets derived from regeneration of petiole explants successfully survived and showed healthy growth when acclimatized in garden soil and transferred to the green house with 86.0 ± 0.9% success rate.

4. Plantlets obtained from induction of somatic embryos (64.2 ± 0.2%) and germination of synthetic seeds from micro shoots (73.0 ± 0.4%) and somatic embryos (70.0 ± 0.1%) of *Gerbera* also showed healthy growth when acclimatized in the green house. Irradiated plantlets at 20 Gy and 30 Gy showed survival rate of 40.1 ± 0.1% and 15.0 ± 0.5%. Meanwhile, only 4.4 ± 0.6% of irradiated plantlets at 40 Gy survived when transferred to the green house.

5. *Gerbera jamesonii in vitro* plantlets grew well and showed good growth performance and development process when acclimatized both in the culture room with temperature of 25 ± 1^{o}C and in the green house with higher temperature of 28 ± 1 ^{o}C. However, flowering only occurred when 6-month-old *in vitro* plantlets were acclimatized in the green house.

6. *In vivo* plants showed higher chlorophyll content compared to *in vitro* acclimatized plantlets.

7. *Gerbera* plantlets grown in the green house exhibited the same morphological characters except for some dissimilarity in plant height and leaf size. Flowers produced were actinomorphic. Differences were observed in peduncle length, number of flower petals, number of flowers per plant and flower size between *in vivo* plants and acclimatized *in vitro* plantlets.

8. Height of *in vivo Gerbera* plant was taller (24.4 ± 0.3 cm) compared to acclimatized *in vitro* plantlets (22.6 ±0.5 cm) and acclimatized *in vitro* plantlets irradiated at 20 Gy (19.0 ± 1.2 cm) , 30 Gy (17.8 ± 0.7 cm) and 40 Gy (17.1 ± 0.4 cm).

9. *In vivo* plants and acclimatized *in vitro* plantlets produced rutted, thick and coarse leaf with silky leaf hair. Meanwhile, acclimatized irradiated plantlets produced rutted, thinner, coarse leaf with fine silky hair.

10. The leaves sizes of *in vivo* plant (3rd leaf from new shoot) were bigger compared to acclimatized *in vitro* and irradiated *in vitro* plantlets. *In vivo* plant produced leaves with 21.3 ± 0.2 cm length and 8.2 ± 0.5 cm width. Acclimatized *in vitro* plantlet produced leaves with 19.3 ± 0.6 cm length and 7.1 ± 0.5 cm width. Meanwhile, acclimatized irradiated plantlet at 20 Gy produced leaves with 15.4 ± 0.7 cm length and 4.6 ± 1.0 cm width, 30 Gy produced leaves with 12.9 ± 0.5 cm length with 3.7 ± 1.3 cm width and 40 Gy produced leaves with 12.0 ± 1.0 cm length and 3.3 ± 0.4 cm width.

11. *Ex-vitro* flowering of acclimatized *in vitro* plantlets was successfully achieved after 6 months of acclimatization process in the green house.

12. Peduncle length of *in vivo* plant was slightly higher (29.7 ± 0.5 cm) compared to acclimatized *in vitro* plantlets (28.6 ± 0.2 cm). Irradiated *in vitro* plantlets were unable to flower in the green house.

13. Numbers of flowers per plant and flower sizes (diameter) of in vivo plant were larger than acclimatized *in vitro* plantlets. *In vivo* plants produced 2.8 ± 0.1 flowers per plant compared to 2.2 ± 0.3 for acclimatized *in vitro* plantlets. Diameter for *in vivo* flower was 8.2 ± 0.4 cm and acclimatized *in vitro* plantlet produced flower with the size of 7.6 ± 0.1 cm.