CHAPTER 3

CALLUS INDUCTION FROM VARIOUS EXPLANTS OF *Gerbera jamesonii* Bolus ex. Hook f.

3.1 EXPERIMENTAL AIMS

Callus is an unorganized, proliferated mass of differentiated plant cells. Callus is usually composed of unspecialized parenchyma cells (Halperin, 1969). Response of callus normally occurred in nature to cover wounded areas of plant tissues. Skoog and Miller (1957); Skoog and Armstrong (1970) and Akiyoshi *et al.*, (1983) demonstrated the importance of growth hormones (auxins and cytokinins) in the plant culture media. This is important in plant tissue culture as it successfully initiated cell division and callus formation.

In tissue culture, cells from small segments of plant organs undergo repeated division to form masses of unorganized cells. Some callus generally undergo differentiation to form shoots and roots and also plantlets through a series of subculturing into suitable regeneration media. Callus cultures are important in plant biotechnology. Manipulation of cytokinin to auxin ratio in the culture medium could lead to the development of shoots, roots and somatic embryos from which, a whole plant could be subsequently produced. Cytokinins are known to promote enlargement of cells in certain plant tissues (Rayle *et al.*, 1982 and Ross and Rayle, 1982), while auxins play important roles in regulating cell elongation, division and differentiation (Dietz *et al.*, 1990).

In exceptional conditions and sometimes spontaneously, the regeneration of adventitious organs or embryos can occur from a callus (Pierik, 1987). In a liquid medium callus may form aggregates which are clumps of cells or individual cells. Plants have the ability to regenerate from single cells. If plant regeneration from callus is required, callus should be inoculated and subcultured into another medium. Normally, organ formation is obtained on a solid medium. All types of organs (root, stem, leave and flower parts etc.) and tissues can be used as starting materials for callus induction. The addition of growth regulators also plays an important role in the initiation and induction of callus. The combination of 2,4-D and coconut milk was effective in inducing callus formation in Carrot (Steward *et al.*, 1958). Coconut milk was later substituted with other types of cytokinin like kinetin. Ruan *et al.* (2009) reported that, callus of *Kosteletzkya virginica* was successfully initiated when embryonic axes were cultured on MS medium supplemented with 1.0 mg/l IAA and 0.3 mg/l kinetin. Other factors that influence the initiation of callus are genotype, nutrient medium, physical factors such as light and temperature.

The main objective of this study was to identify the optimum media in establishment of callus from *Gerbera* leaf and petiole explants. Growth and development of callus were observed. The content of secondary metabolite was also studied to identify the types of compounds contained in *Gerbera* callus. Identification of valuable secondary metabolites in callus of *G. jamesonii* was important since mass proliferation of callus could produce large amount of valuable compounds. Studies on establishment of callus for induction of somatic embryos will be further investigated in chapter 4.

3.2 MATERIALS AND METHODS

3.2.1 Seed Sterilization

Seeds of *Gerbera jamesonii* were sterilized (refer to 2.2.4) and cultured on MS basal medium supplemented with 30 g/l sucrose and 0.8% technical agar. The medium was autoclaved for 21 minutes at a pressure of 104 kPa (15 Psi^2) and temperature of 121 ^oC. All cultures were maintained in the culture room at 25 ± 1 ^oC and 16 hours light and 8 hours dark.

3.2.2 Callus Induction

Young aseptic seedlings were utilized as explant sources after 4 weeks in culture. Secondary leaves and petioles were used to initiate cultures for callus induction. The leaves and petioles were cut into 10 mm x 10 mm square and respectively cultured on MS medium with 3% sucrose and 0.8% technical agar. Different concentrations of BAP and 2, 4-D were added in the culture medium. Based on experiments in chapter 2, combinations of BAP and 2, 4-D in the culture medium were found to be the most responsive hormones in the induction of callus. Therefore, in this experiment, various concentrations and combinations of BAP and 2, 4-D were used as follows;

1. 1.0 mg/l BAP + 0.1 mg/l 2,4-D
2. 1.0 mg/l BAP + 0.5 mg/l 2,4-D
3. 1.0 mg/l BAP + 1.0 mg/l 2,4-D
4. 1.0 mg/l BAP + 1.5 mg/l 2,4-D

5. 1.0 mg/l BAP + 2.0 mg/l 2,4-D
6. 1.0 mg/l BAP + 2.5 mg/l 2,4-D
7. 1.0 mg/l BAP + 3.0 mg/l 2,4-D
8. 2.0 mg/l BAP + 0.1 mg/l 2,4-D
9. 2.0 mg/l BAP + 0.5 mg/l 2,4-D
10. 2.0 mg/l BAP + 1.0 mg/l 2,4-D
11. 2.0 mg/l BAP + 1.5 mg/l 2,4-D
12. 2.0 mg/l BAP + 2.0 mg/l 2,4-D
13. 2.0 mg/l BAP + 2.5 mg/l 2,4-D
14. 2.0 mg/l BAP + 3.0 mg/l 2,4-D
15. 3.0 mg/l BAP + 0.1 mg/l 2,4-D
16. 3.0 mg/l BAP + 0.5 mg/l 2,4-D
17. 3.0 mg/l BAP + 1.0 mg/l 2,4-D
18. 3.0 mg/l BAP + 1.5 mg/l 2,4-D
19. 3.0 mg/l BAP + 2.0 mg/l 2,4-D
20. 3.0 mg/l BAP + 2.5 mg/l 2,4-D
21. 3.0 mg/l BAP + 3.0 mg/l 2,4-D

Thirty replicates of explants were used in each treatment. All cultures were maintained in the culture room at 25 ± 1 ^{o}C and 16 hours light and 8 hours dark for 8 weeks. Morphological aspects of the callus were observed based on the quality and quantity. Fresh weight of all callus obtained were recorded.

3.2.3 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

3.2.4 Studies on Secondary Metabolites in Callus

The presence of secondary metabolites in callus of *Gerbera jamesonii* were analyzed through thin layer chromatography (TLC). Pre-layered silica gel plates (Kieselgel 60 PF_{254}, Merck) were dropped with a drop of sample and placed in the chromatography tank that has been saturated with appropriate solvent system for 30 minutes. Six solvent systems were used in this experiment. The solvent systems are;

System	Solvent	Ratio of volume
I	Toluene: Ethyl acetate: Acetic acid	50: 48: 2
II	Toluene: Ethyl acetate: Acetic acid	75: 20: 5
III	Petroleum ether: Ethyl acetate: Formic acid	75: 25: 1
IV	Hexane: Ethyl acetate	30: 70
V	Hexane: Ethyl acetate	50: 50
VI	Hexane: Ethyl acetate	70: 30

The location of separated components were observed under visible light, exposed to iodine vapour and also sprayed with Vanillin-sulphuric acid reagent and Anesaldehyde-sulphuric acid reagent.

The retention factor (R_f) for each component is measured based on:

$$R_f = \frac{\text{Distance traveled by the compounds}}{\text{Distance traveled by the solvent front}}$$

3.3 RESULTS

3.3.1 Callus Induction

In theory, any part obtained from any plant species can be induced to form callus tissue, however the successful production of callus depends upon plant species and their qualities. Dicotyledons are rather amenable for callus tissue induction, as compared to monocotyledons; the callus of woody plants generally grow slowly. Callus induction from leaf and petiole explants have been successfully achieved in *G. jamesonii* Bolus ex. Hook f. The highest fresh weight (78.8 ± 0.8%) was obtained 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, Figure 3.1). 70.5 ± 0.6% of callus was achieved when the culture medium was added with 2.0 mg/l BAP and 1.0 mg/l 2, 4-D while callus fresh weight percentage was reduced to 67.0 ± 0.7% when the concentration of 2, 4-D was reduced to 0.5 mg/l. The lowest fresh weight percentage (18.6 ± 0.3%) was observed when leaf explants were cultured on MS medium supplemented with 3.0 mg/l BAP and 2.5 mg/l 2, 4-D. Green compact callus was formed in all treatments when callus were induced from leaf explant (Table 3.1). It was also observed that the fresh weight percentage reduced when 2, 4-D concentration exceeded 2.0 mg/l.

Induction of callus from petiole explants gave the highest fresh weight percentage when explants were 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 with 70.3 ± 0.5% (Table 3.2,

Figure 3.4) and green compact callus was formed. The fresh weight percentage of callus induced from petiole explants was observed to be slightly lower compared to callus induced from leaf explants. Yellowish green compact callus was observed when 3.0 mg/l BAP was fortified to the culture medium. The results indicated that, leaf explants showed better callus formation compared to petiole explants. Fresh weight percentage of callus was calculated according to the following formula:

Fresh Weight Percentage:

$$\frac{\text{Total fresh weight of callus } (8^{th} \text{ week}) \text{ (mg)} - \text{Total initial fresh weight of explant (mg)}}{\text{Total initial fresh weight of explant (mg)}} \times 100$$

Valuable chemical content and secondary metabolites in callus of *G. jamesonii* were screened through thin layer chromatography (TLC).

Table 3.1: Callus induction from leaf explant of *Gerbera jamesonii* Bolus ex. Hook f. Thirty replicates were used in each treatment.

MS + Hormone (mg/l)		Fresh weight (%)	Observations
BAP (mg/l)	2,4-D (mg/l)		
1.0	0.1	$47.4 \pm 0.5_{c,d}$	Light green compact callus
1.0	0.5	$58.0 \pm 0.9_{c}$	Light green compact callus
1.0	1.0	38.0 ± 0.3	Light green compact callus
1.0	1.5	$63.1 \pm 0.6_{b,c}$	Light green compact callus
1.0	2.0	$78.8 \pm 0.8_{a}$	Light green compact callus
1.0	2.5	$70.0 \pm 0.5_{b}$	Light green compact callus
1.0	3.0	$67.2 \pm 0.4_{b}$	Light green compact callus
2.0	0.1	58.3 ± 0.4	Green compact callus
2.0	0.5	$67.0 \pm 0.7_{b}$	Green compact callus
2.0	1.0	$70.5 \pm 0.6_{b}$	Green compact callus
2.0	1.5	$58.4 \pm 0.2_{c}$	Green compact callus
2.0	2.0	$50.5 \pm 0.4_{c}$	Green compact callus
2.0	2.5	$48.0 \pm 0.5_{c,d}$	Green compact callus
2.0	3.0	$41.6 \pm 0.5_{d}$	Green compact callus
3.0	0.1	$26.0 \pm 0.4_{e}$	Green compact callus
3.0	0.5	$27.4 \pm 0.6_{e}$	Green compact callus
3.0	1.0	$24.0 \pm 0.4_{e}$	Green compact callus
3.0	1.5	$20.3 \pm 0.5_{e}$	Green compact callus
3.0	2.0	$22.5 \pm 0.6_{e}$	Green compact callus
3.0	2.5	$18.6 \pm 0.3_{e,f}$	Green compact callus
3.0	3.0	$19.2 \pm 0.5_{e,f}$	Green compact callus

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

Table 3.2: Callus induction from petiole explant of *Gerbera jamesonii* Bolus ex. Hook f. Thirty replicates were used in each treatment

MS + Hormone (mg/l)		Fresh weight (%)	Observations
BAP (mg/l)	2,4-D (mg/l)		
1.0	0.1	$35.6 \pm 0.3_{d}$	Green compact callus
1.0	0.5	$42.0 \pm 0.6_{c,d}$	Green compact callus
1.0	1.0	$40.6 \pm 0.9_{c,d}$	Green compact callus
1.0	1.5	$52.7 \pm 1.1_{c}$	Green compact callus
1.0	2.0	$70.3 \pm 0.5_{a}$	Green compact callus
1.0	2.5	$63.3 \pm 0.5_{b,c}$	Green compact callus
1.0	3.0	$59.7 \pm 0.4_{c}$	Green compact callus
2.0	0.1	$39.5 \pm 1.2_{d}$	Green compact callus
2.0	0.5	$45.2 \pm 0.7_{c,d}$	Green compact callus
2.0	1.0	$66.9 \pm 0.6_{b}$	Green compact callus
2.0	1.5	$68.3 \pm 0.2_{b}$	Green compact callus
2.0	2.0	$63.1 \pm 1.0_{b,c}$	Green compact callus
2.0	2.5	$68.0 \pm 0.2_{b}$	Green compact callus
2.0	3.0	$61.2 \pm 1.3_{b,c}$	Green compact callus
3.0	0.1	$24.2 \pm 0.8_{e}$	Green compact callus
3.0	0.5	$26.0 \pm 0.5_{e}$	Yellowish green compact callus
3.0	1.0	$29.6 \pm 0.4_{e}$	Yellowish green compact callus
3.0	1.5	$20.2 \pm 1.0_{e}$	Yellowish green compact callus
3.0	2.0	$13.0 \pm 1.1_{e,f}$	Yellowish green compact callus
3.0	2.5	$17.4 \pm 0.8_{e,f}$	Yellowish green compact callus
3.0	3.0	$12.6 \pm 0.5_{e,f}$	Yellowish green compact callus

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

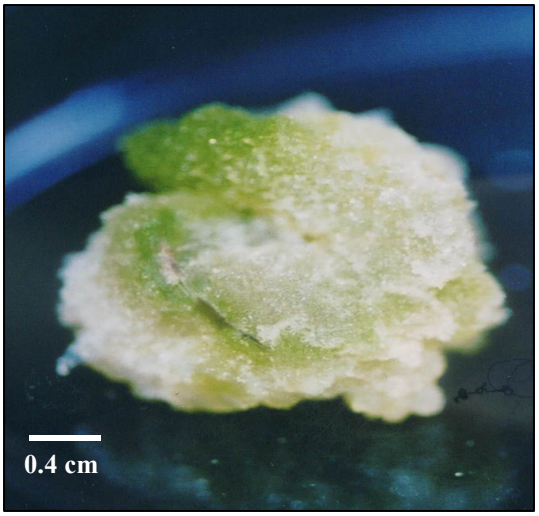

Figure 3.1: Callus derived from leaf explant cultured on MS medium supplemented with 2.0 mg/l 2, 4-D and 1.0 mg/l BAP.

Figure 3.2: Callus derived from leaf explants cultured on MS medium supplemented with 1.0 mg/l 2, 4-D and 2.0 mg/l BAP.

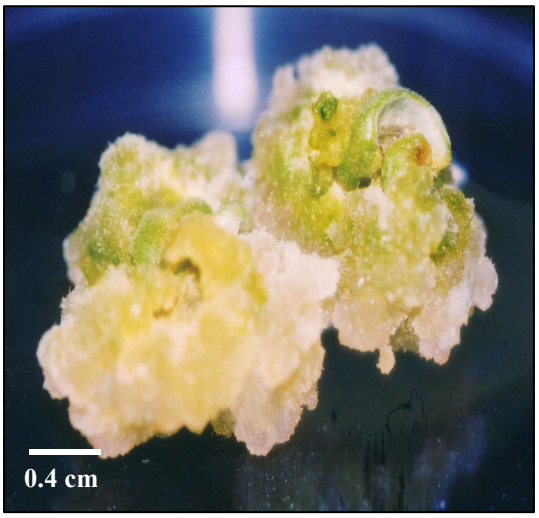

Figure 3.3: Callus derived from leaf explant cultured on MS medium supplemented with 0.5 mg/l 2, 4-D and 2.0 mg/l BAP.

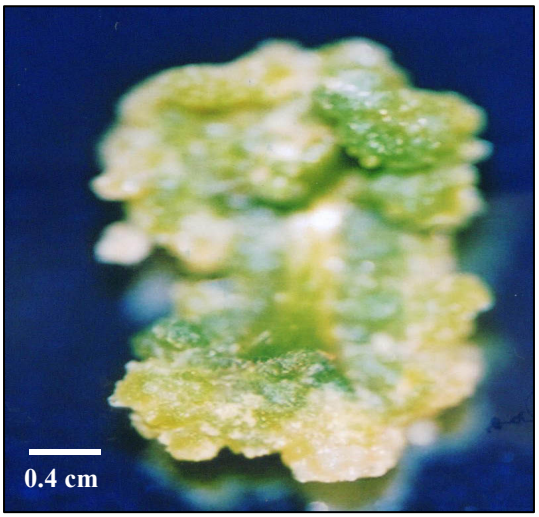

Figure 3.4: Callus derived from petiole explant cultured on MS medium supplemented with 2.0 mg/l 2, 4-D and 1.0 mg/l BAP.

3.3.2 Screening of Secondary Metabolites

Six different solvent systems were used in the study concerning screening of secondary metabolites. Methanol extract of *Gerbera* callus were used as sample (Table 3.3). Samples were tested with Vanillin-sulphuric acid reagent and Anesaldehyde-sulphuric reagent and also exposed to iodine vapour. Brown reddish spot was observed when samples were exposed to iodine vapour. This result showed that callus of *G. jamesonii* contained conjugated chain compound.

Red spot was observed when saturated sample in chromatography tank was sprayed with anesaldehyde-sulphuric acid reagent. The most suitable solvent system used was toluene: ethyl acetate: acid acetic at 50: 48: 2 ratios. The retention factor value (Rf) calculated was 0.85. The presence of red spot showed that *Gerbera* callus contained flavonoid.

Meanwhile, purple spot was observed when sample was tested with vanillin-sulphuric acid reagent. The most suitable solvent system for this test was toluene: ethyl acetate: acid acetic at 75: 20: 5 ratios with Rf value of 0.81. The results showed that callus of *G. jamesonii* also contain terpenoid.

Table 3.3: Thin layer chromatography (TLC) of methanol extract of callus of *Gerbera jamesonii* Bolus ex. Hook f.

Solvent System	**Rf**		**Iodine Vapour**	**Spray Reagent**		**Observations**
	A (anesaldehyde)	**V (vanillin)**		**Anisaldehyde**	**Vanillin**	
Toluena:EA: AA 50: 48: 2	0.85	0.78	Brown-reddish spot ++	Red spot ++	Purple spot ++	Red spot-flavonoid Purple spot- terpenoid
Toluena: EA: AA 75: 20: 5	0.70	0.81	Brown-reddish spot ++	Red spot +++	Purple spot +++	Red spot-flavonoid Purple spot-terpenoid
PE: EA: AF 75: 25: 1	0.58	0.62	Brown-reddish spot ++	Red spot +++	Purple spot +++	Red spot-flavonoid Purple spot-terpenoid
Heksana: EA 30: 70	0.50	0.55	Brown-reddish spot ++	Red spot ++	Purple spot ++	Red spot-flavonoid Purple spot-terpenoid
Heksana: EA 50: 50	0.36	0.38	Brown-reddish spot ++	Red spot ++	Purple spot ++	Red spot-flavonoid Purple spot-terpenoid
Heksana: EA 70: 30	0.24	0.20	Brown-reddish spot ++	Red spot ++	Purple spot ++	Red spot-flavonoid Purple spot-terpenoid

Key:

EA = Ethyl acetate
AA = Acetic acid
PE = Petroleum ether
FA = Formic acid

Colour intensity:

\+ = weak
++ = medium
+++ = strong

3.4 SUMMARY OF RESULTS

1. Leaf explants of *Gerbera jamesonii* exhibited better percentage of callus formation compared to petiole explants.

2. The optimum percentage of callus formation (78.8 ± 0.8%) was observed when leaf explants were cultured on MS medium supplemented with 1.0 mg/l BAP and 2.0 mg/l 2, 4-D. Green compact callus was induced.

3. The lowest percentage of callus (12.6 ± 0.5%) was observed when petiole explants were cultured on MS medium supplemented with 3.0 mg/l BAP and 3.0 mg/l 2, 4-D. Yellowish green compact callus was induced.

4. Screening of secondary metabolites in callus of *Gerbera jamesonii* was carried out using thin layer chromatography (TLC). It was found that callus of *G. jamesonii* contained flavonoid, terpenoid and conjugated chain compounds.