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Identification of Embryogenic Callus and *in vitro* Somatic Embryo Formation in *Gerbera jamesonii* Bolus ex. Hook f.

Nor Azlina Hasbullah[1], Rosna Mat Taha[1] and Asmah Awal[2]
[1]Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
[2] MARA University of Technology, Negeri Sembilan Branch, 72000 Kuala Pilah, Negeri Sembilan, Malaysia.

ABSTRACT

In vitro cultures of *Gerbera jamesonii* Bolus ex. Hook f. were initiated from leaf explants. White friable callus was formed after 4 weeks at an average of 70% on Murashige and Skoog (MS) medium supplemented with 2, 4 dichlorophenoxyacetic acid (2,4-D). The identification of somatic embryos at early stages was done by treatment with 2% Acetocarmine and 0.5% Evan's Blue (double staining method). The embryogenic head cells stained bright red (acetocarmine) and suspensor cells stained blue in an embryogenic mass. In the mass of non-embryogenic callus, cells did not show any organization of heads and suspensors and will stain blue with Evan's Blue. White-cream friable embryogenic callus of *Gerbera jamesonii* was formed after 4 weeks when leaf explants were cultured on MS medium supplemented with 0.1 mg/l-2.0 mg/l 2, 4-D, 3.0% sucrose and solidified with 0.8% agar. The embryogenic callus was transferred into MS suspension culture medium supplemented with 1.0 mg/l 2, 4-D and 0.1 mg/l Naphthalene acetic acid (NAA) and was subcultured at 10 days interval for 1 month. Subsequent withdrawal of 2, 4-D from induction medium resulted in the induction and growth of somatic cells. Somatic embryos formed at globular phase were then sieved and transferred into maturation medium. Heart, torpedo and cotyledon phases of somatic embryo were identified.
Keywords: *Gerbera jamesonii*, embryogenic callus, non-embryogenic callus, somatic embryo.

INTRODUCTION

Gerbera jamesonii Bolus ex. Hook f., is commonly known as *Gerbera* daisy or Barberton Daisy. This temperate perennial flowering plant belongs to the Asteraceae family. *Gerbera* are well known as a beautiful ornamental plant with very high commercial values. They are planted in full sun and useful as cut flowers, pot plant and also bedding plant. Plant propagation through tissue culture of *Gerbera* is mainly aimed to produce plants at a very high multiplication rate since this plant is highly demanded all around the world. *Gerbera* are well known as a beautiful ornamental plant with very high commercial values. Through tissue culture techniques, *Gerbera* shoots were regenerated primarily from flower buds from greenhouse grown plants (Pierik *et al.*, 1973, 1975; Laliberte *et al.*, 1985). As an alternative to *in vitro* propagation, somatic embryogenesis was introduced. Hundreds of new plantlets could be formed from small pieces of explants through somatic embryogenesis. Somatic embryogenesis is originally induced from somatic tissues or vegetative tissues which are not involved in the production of plant through natural breeding. To induce somatic embryogenesis in *Gerbera jamesonii*, explants need to be cultured on selected medium. Embryogenic callus formed, will then be transferred to suspension medium. When the culture conditions of the callus meet the optimum requirements, cells will form embryo structures such as globular, heart, torpedo and cotyledon. These embryo stages are similar to the embryos formed from zygotic cells.

Embryogenic callus from *Gerbera jamesonii* were induced and four phases of somatic embryo were formed. However, it is difficult to differentiate embryogenic cells from non-embryogenic cells. A double staining technique has been developed to distinguish embryogenic cells from non-embryogenic ones (Gupta and Durzan, 1987). This method is called double staining which involved two stains, acetocarmine and Evan's blue. Using this staining method, embryogenic cells can easily be indentified. The present study was mainly aimed to produce somatic embryos from *Gerbera jamesonii* and to identify, differentiate embryogenic cells of *Gerbera* callus through double staining method.

MATERIALS AND METHODS

Source of Explant

Gerbera seeds were soaked in distilled water for 30 minutes with addition of 1-2 drops of Tween-20 followed by 40% (v/v) sodium chloride solution and gently agitated for 10 minutes. The seeds were then rinsed 3 times in distilled water and then soaked in 70% (v/v) alcohol for 1 minute. Finally, the seeds were rinsed 3 times in sterile distilled water. Sterilized seeds were cultured on MS (Murashige and Skoog, 1962) basal medium. pH of the medium was adjusted to 5.8 before being autoclaved at 121^{o}C for 21 minutes. Leaves obtained from aseptic young plantlets (8-weeks-old) were used as source of explants.

Embryogenic Callus Initiation and Establishment of Cell Suspension Culture

The secondary leaves of seedlings were cultured on MS medium supplemented with 0.1- 2.0 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D), 3.0% sucrose and 0.8% agar and incubated in the dark at 25^{o}±1^{o}C for 2

* Corresponding Author: azahhas@yahoo.com

months (Table 1). After 2 months, white friable callus was transferred into somatic embryo induction suspension medium, 0.1-2.0mg/l 2, 4-D and 0.1 or 1.0mg/l α-Naphthalene acetic acid (NAA) with 3.0% sucrose (Table 1). Ten 125 ml flasks containing 30ml of the medium and 2.0g of callus tissue were used in two replications. All cultures were incubated on a gyratory shaker at 110rpm and maintained in the dark at 25°±1°C. The cultures were subcultured at 10 days interval for 4 to 6 weeks.

Non-Embryogenic Callus Production

Secondary leaves of aseptic seedlings were used to initiate non-embryogenic callus. Explants were cultured on MS medium medium supplemented with 1.0mg/l 6-benzylaminopurine (BAP) and 1.0mg/l NAA with 3.0% sucrose and 0.8% agar. Cultures were incubated at 25°±1°C at 16 hours light and 8 hours dark.

Induction of Somatic Embryos

The suspension cells were then filtered through 425µm pore size woven wire test sieve to separate the cell clumps. The filtrate was rinsed with plain liquid MS medium. The cell clumps were then transferred into 25 petri dishes containing MS medium supplemented with 0.1-1.0mg/l BAP and 0.1-1.0mg/l NAA with the addition of 0 or 50mM Proline in each treatment (Table 1). All cultures were incubated in 16 hours light and 8 hours dark at 25°C. Cultures were observed for 4-6 weeks. Data were analyzed using analysis of variance and all means were compared using paired Duncan's Multiple Range Test.

Identification of Embryogenic Callus

A small piece of callus was placed on a glass slide and 2-3 drops of 2% acetocarmine solution was dropped onto the callus. The callus were divided into small pieces and heated over a low flame for a few seconds. The slide was rinsed with distilled water to remove all liquid. 2-3 drops of 0.5% of Evan's blue solution was dropped to acetocarmine stained cells. After 30 seconds, the slide was rinsed again with distilled water and all excessive water was removed. 1-2 drops of glycerol was added to the stained cells, in order to prevent the cells from drying.

RESULTS

White-cream friable callus was formed after 6 weeks when leaf explants were cultured on MS medium supplemented with 0.01-2.0mg/l 2, 4-D. Green coloured callus was formed when the same explants were cultured on 1.0mg/l BAP and 1.0mg/l NAA. Young secondary leaf from aseptic seedling was identified as the best explant for the induction of embryogenic callus and somatic embryo. These callus were examined using double staining method to determine the embryogenic and non-embryogenic characters. Under the microscope observation, white-cream friable callus showed early stage embryos, the embryonal heads stained red (Fig. 1a) and suspensors stained blue. Meanwhile, when the green callus was double stained, the cells remained blue and did not show any organization of head and suspensor (Fig. 1b).

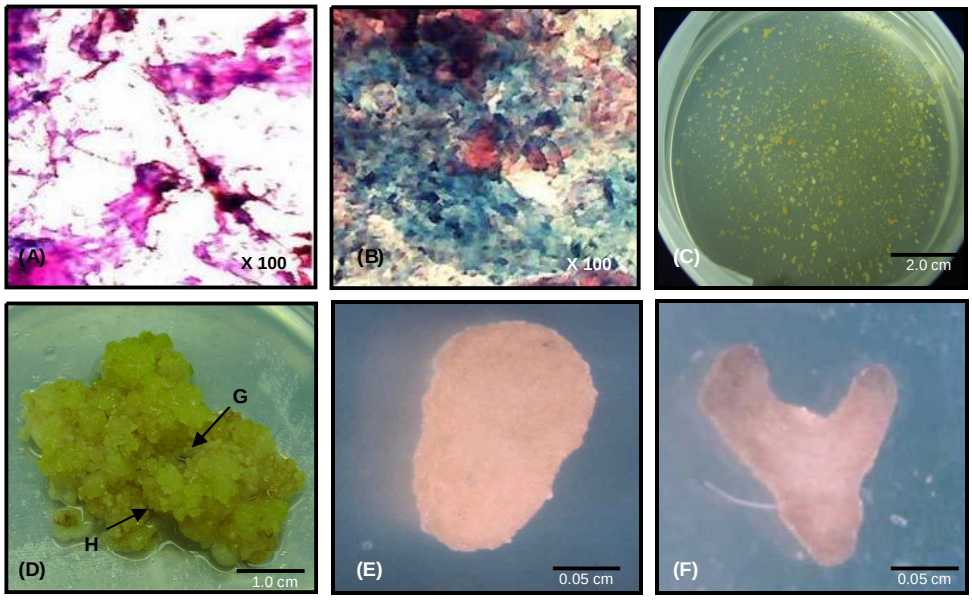

Figure (1): (A) Early Stage of embryos after double staining, embryogenic cells are stained red (acetocarmine), **(B)** Non-embryogenic cells are stained blue with Evan's blue stain, **(C)** Somatic embryogenesis of *G. jamesonii* from suspension culture, **(D)** Embryogenic callus after two weeks showing globular and heart stages of somatic embryogenesis, **(E)** Globular shaped somatic embryo of *G. jamesonii*, and **(F)** Heart shaped somatic embryo.

Table (1): Composition of culture medium and growth condition for *Gerbera jamesonii* somatic embryo induction.

Culture stage	Culture medium	Growth Regulators (mg/L)			L-Proline (mM)	Culture duration (week)
		BAP	NAA	2,4-D		
Seedling	MS	-	-	-	-	8
Callus induction	MS	-	-	0.1-2.0	-	6
Cell suspension	MS	-	0.1	1.0	-	1
Embryo induction	MS	0.1-2.0	0.1-1.0	-	0-50	3
Conditioning phase	MS	-	-	-	-	3
Root growth	MS	-	-	-	-	2

The addition of 2, 4-D at concentration of 0.01-2.0mg/l in the culture medium has initiated the growth of embryogenic callus. All callus formed was friable and white-cream coloured. No callus was formed when explants were cultured on MS basal medium. Embryogenic callus was obtained (100%) when explants were cultured on MS medium supplemented with 1.8mg/l or 2.0mg/l 2, 4-D with the addition of 30% sucrose and 0.8% technical agar (Table 2). Production of embryogenic callus was decreased as 2, 4-D concentration in medium decreased.

These embryogenic callus 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. All cultured callus began to dissociate into single cells and small cell clumps within the period. Embryogenesis was observed after 1 week in cell suspension. Cell clusters and aggregates from cell suspension culture were observed (Fig. 1c). Cells were sieved and transferred into embryo induction medium containing BAP at concentrations from 0-2.0mg/l in combination with 0.1 or 1.0mg/l NAA with the addition of 0 or 50 mM L-Proline (Table 3). During this phase, stages of somatic embryos were developed (Fig. 1d). Embryogenic callus which has recovered from suspension medium started to develop on agar solidified medium. Three weeks after the transfer of embryogenic callus to the embryo induction medium, 15.7 ± 1.4 embryos were developed in culture medium contained 1.0mg/l BAP and 0.1mg/l NAA without the addition of L-proline (Table 3). Meanwhile, medium supplemented with the same concentration of growth regulators, with the addition of 50mM L-proline produced the highest embryo yield (29.8 ± 1.2 embryos). Morphological observation of the embryo stages was done and stages from globular, heart, torpedo and cotyledon were successfully identified as shown in Fig. 1D, 1F and Fig. 2A, 2B. Addition of L-proline in the induction medium promoted the development of somatic embryos to form shoots and plantlets. All embryos were then transferred to MS basal medium for further development of plantlets and root growth (Fig. 2C). Plantlets formed were then transferred to soil and grown in the green house for further growth and development (Fig. 2D, E).

Table (2): Effects of 2, 4-D on percentage of embrogenic callus formation of *G. jamesonii* Basic medium used was MS.

2,4-D (mg/l)	Callus Formation (%)
0.0	0.0
0.01	49.7 ± 1.1
0.1	65.7 ± 3.0
0.5	69.3 ± 1.7
1.0	73.1 ± 0.9
1.5	85.0 ± 0.6
1.8	100
2.0	100

Table (3): Effects of BAP, NAA and L-proline on formation of somatic embryos of *Gerbera jamesonii* Bolus ex. Hook f. in suspension culture.

BAP (mg/l)	Number of Somatic Embryos			
	NAA (mg/l)		NAA (mg/l) + 50 mM L-Proline	
	0.1	**1.0**	**0.1**	**1.0**
0.0	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ±0.0 a	0.0 ± 0.0a
0.1	5.2 ± 0.3b	0.0 ± 0.0a	9.7± 1.5b	0.0 ± 0.0a
0.5	8.0 ± 1.0b	0.0 ± 0.0a	21.3 ± 0.8d	0.0 ± 0.0a
1.0	15.7 ± 1.4c	3.3 ± 0.1b	29.8 ± 1.2d	11.6 ± 0.9bc
2.0	10.4 ± 0.5bc	6.2 ± 0.4b	18.5 ± 1.7c	7.2 ± 1.1b

Mean ± SE, n=20. Mean with different letters differ significantly at p=0.05

DISCUSSION

Somatic embryogenesis is a powerful tool for plant propagation and improvement of ornamental plant. It is controlled by *in vitro* and *in vivo* environmental variables (Ammirato, 1983). Embryogenic callus that promoted the production of somatic embryos were successfully induced in *Gerbera jamesonii* bolus ex. Hook f. In order to differentiate embryogenic cells from organogenic cells, double staining method was used. Embryogenic cells are small in size, have large nuclei, small vacuoles with densed cytoplasm. It is also known to have a high proliferation potential (Pierik, 1987). These nuclei were stained bright red with acetocarmine. Strands in the cytoplasm also showed an affinity for acetocarmine and stained bright red. Acetocarmine is usually used to detect DNA, chromatin and glycoproteins in cytochemical studies (Sharma and Sharma, 1980). To further differentiate the embryogenic mass, smaller nuclei, which are associated with the formation of suspensors derived from the embryogenic cells, reacted with Evan's blue. The exclusion of Evan's

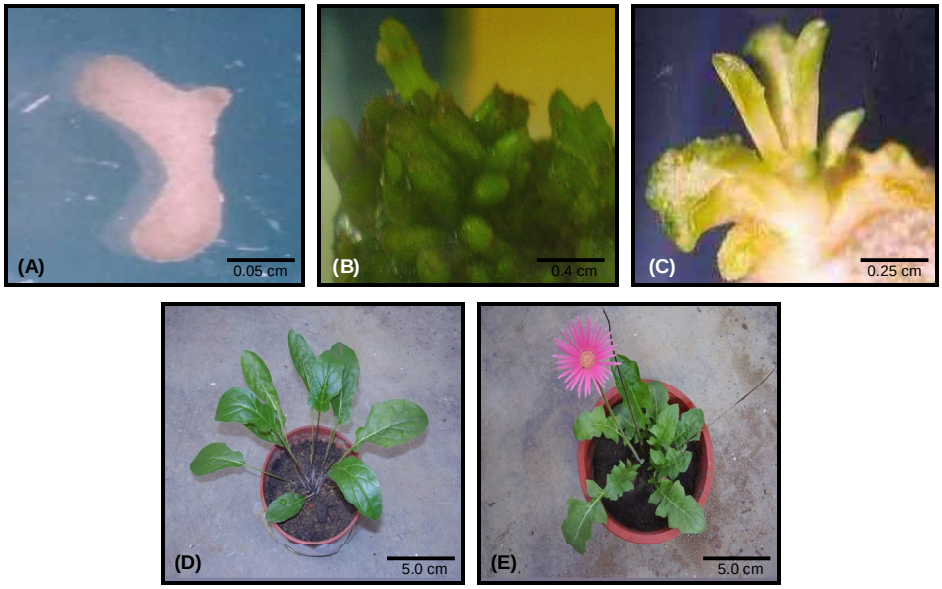

Figure (2): (A) Torpedo shaped somatic embryo of *Gerbera jamesonii*, **(B)** Mature cotyledonary stage somatic embryo, **(C)** Microshoots produced from somatic embryo, **(D)** Plantlet produced from somatic embryo two months after transferred to soil, and **(E)** Flowering plant produced from somatic embryo after 6 months being transferred to soil.

blue determines the viability of cells. Less viable cells are vacuolated with small nuclei that permitted Evan's blue dye to enter. However, in non-embryogenic callus, which has small nuclei, red stain of acetocarmine was difficult to locate and the whole cells stained blue with Evan's blue.

Induction of somatic embryogenesis requires a modification and change in the vegetative cells. In *Gerbera*, the most common responsive treatment is MS medium, supplemented with 2, 4-D at a lower concentration. However, other auxins such as NAA were also effective. The addition of very low concentration of NAA also promoted somatic embryogenesis.

The results also showed that L-proline played an important role in the production of somatic embryos. The present work reported for the first time, somatic embryos in *Gerbera* were obtained using the combination of BAP, NAA and L-proline. The above hormones proved to be very effective for the induction of somatic embryos. 29.8 ± 1.2 of somatic embryos were successfully obtained when explants were cultured on MS medium supplemented with 1.0 mg/l BAP, 0.1 mg/l NAA and the addition of 50mM L-proline. A determining role of L-proline in the tissues culture of other plants and its positive role on embryo formation in corn were also noted (Armstrong and Green, 1985; Suprasanna *et al.*, 1994). The physiological roles of proline in the enzyme regulation of plants in stress condition were also discussed (Stewart and Boggess, 1977) and its effect on carbon and nitrogen storage has been explained (Jager and Meyer, 1977). The presence of L-proline in the culture medium produced a required stress condition by decreasing water potential level in plant cell culture medium and at the same time, nutritional elements in the cells were increased. Thus, somatic embryogenesis was enhanced. Embryogenic callus and somatic embryos of African marigold (*Tagetes erecta* L.) were induced when cotyledonary explants were cultured on MS medium supplemented with 2.0 mg/l 2, 4-D and 0.2 mg/l Kinetin (Bespalhok and Hattori, 1998). Although somatic embryogenesis has been described for more than a hundred plant species from different families (Terzi and Loschiavo, 1990), the number of reports among members of the Asteraceae family is still low (May and Trigiano, 1991). Somatic embryos formed from embryogenic callus of *Gerbera* were transferred to MS basal medium for shoot formation and root elongation. Plantlets produced were successfully transferred to soil and maintained in the green house for further growth.

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REFERENCES

AMMIRATO, P.V. 1983. Embryogenesis; In the handbook of plant cell culture techniqes for propagation and breeding, D.A. Evans., W.R. Sharp., P.V. Ammirato and Y. Yamada (eds.), New York,

U.S.A.: Macmillan Publishing Company **1**: 82-123

ARMSTRONG, C.L., AND C.E. GREEN. 1985. Establishment and maintanence of friable, embryogenetic maize callus and the involvement of L-proline. Planta **164**: 207-214.

BESPALHOK, J.C.F., AND K. HATTORI. 1998. Friable embryogenic callus and somatic embryo formation from cotyledon explants of African marigold (*Tagetes erecta* L.). Plant Cell Reports **17**: 870-875.

GUPTA, P.K., AND D.J. DURZAN. 1987. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Biotechnology **5**: 147-151.

JAGER, H.J., AND H.R. MEYER. 1977. Effect of water stress on growth and praline metabolism of *Phaseolus vulgaris* L. Oecologia **30**: 83-96.

LALIBERTE, S., L. CHRETIEN, AND J. VIETH. 1985. *In vitro* plantlet production from young capitulum explants of *Gerbera jamesonii*. Horticultural Science **20**: 137-139.

MAY, R.A., AND R.N. TRIGIANO. 1991. Somatic embryogenesis and plant regeneration from leaves of *Dendrathema grandiflora*. Journal of American Society for Horticultural Science **116**: 366-371.

MURASHIGE, T., AND F. SKOOG. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Plant Physiology **15**: 473-497.

PIERIK, R.L.M. 1987. *In vitro* culture of higher plants. Martinus Nijhoff Publishers, Dordrecht, the Netherlands.

PIERIK, R.L.M., J.L.M. JANSEN, A. MAASDAM, AND C.K. BINNENDIJK. 1975. Optimalization of *Gerbera* plantlet production from excised capitulum explants. Scientia Horticulturae **3**: 351-357.

PIERIK, R.L.M., H.H.M. STEEGMANS, AND J.J. MARELIS. 1973. *Gerbera* plantlets from *in vitro* cultivated capitulum explants. Scientia Horticulturae **1**: 117-119.

SHARMA, A.K., AND A. SHARMA. 1980. Chromosome techniques, theory and practice. Frakenham Press, Ltd., Norfolk.

STEWART, C.R., AND S.F. BOGGESS. 1977. The effect of wilting on the conversion of arginine, ornithine, and glutamate to praline in bean leaves. Plant Science Letters **8**: 147-153.

SUPRASANNA, P., K.V. RAO, AND G.M. REDDY. 1994. Embryogenic callus in maize: Genotypic and amino acid effects. Cereal Res Communication **22**: 79-82.

TERZI, M., AND F. LOSCHIAVO. 1990. Somatic embryogenesis. In: Bhojwani SS (Ed.). Development in crop science 19: plant tissue culture: applications and limitations. Elsevier, Tokyo.

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