

Full Length Research Paper

Establishment of somatic embryogenesis from *Gerbera jamesonii* Bolus EX. Hook F. through suspension culture

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Cell suspension cultures were established from embryogenic callus induced from leaf explants of *Gerbera jamesonii* Bolus ex. Hook f. Embryogenic callus was induced when leaf explants were cultured on MS medium containing 1.0 to 2.0 mg/L 2,4-D. Cream friable callus was formed within two weeks. Proliferated callus was transferred to MS liquid medium containing 2,4-D with a small concentration of NAA and subcultured at 2 weeks interval. Induction of somatic embryos (globular, heart and torpedo) was observed after 2 weeks of culture. Somatic embryos developed in MS suspension medium containing 1.0 to 2.0 mg/L 2,4-D with 0.1 or 1.0 mg/L NAA and globular embryos were further differentiated into the cotyledonary phase embryos. The addition of amino acids (L-glutamine or L-proline, 5.0 mg/L, respectively) to the culture media, in the range of concentrations tested, yielded higher enhancement of the embryo growth and development. Transfer of individual embryos onto a fresh basal medium with no plant growth regulators was able to achieve complete maturation. Relatively, only a few number of embryos developed shoots and roots when transferred to MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA in addition to 3% (w/v) sucrose and 0.8% (w/v) agar containing medium. About 11% of somatic embryos were converted to true-to-type fertile plants.

Key words: *Gerbera jamesonii*, embryogenic callus, cell suspension culture, plant growth regulators, amino acid.

INTRODUCTION

Gerbera jamesonii Bolus ex. Hook f. known as 'Barberton Daisy' or 'Transvaal Daisy' is a member of the Asteraceae family, characterized by its rosette shape. *Gerbera* is an evergreen temperate plant. However, through proper growing techniques, this plant species has successfully be adapted to tropical countries due to the prevalent economic and ecological conditions. Since this plant is a well known ornamental plant with very high commercial and economical values, interest in it has recently heightened in breeding good cut flowers, potted

as well as bedding plant. In tissue culture studied, plant regeneration was uniformly achieved when different explants were used as source of plant material (Murashige, 1977). Plant propagation through tissue culture of *G. jamesonii* is mainly aimed to produce plants at a very high multiplication rate since this plant is highly demanded all around the world. Through tissue culture techniques, *Gerbera* shoots were regenerated primarily from floral bud explants from greenhouse grown plants (Pierik et al., 1973, 1975; Laliberte et al., 1985; Posada et al., 1999; Mohammed and Ozzambak, 2007), leaves (Hedtrich, 1979; Barbosa et al., 1994), floral bracts (Maia et al., 1983), torus (Zhang, 2002), inflorescence (Schum and Busold, 1985; Misra et al., 2010) and petiole explants from aseptic seedling (Hasbullah et al., 2008). As an alternative to *in vitro* propagation, somatic embryogenesis was introduced. Somatic embryogenesis is originally induced from somatic tissues or vegetative

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Abbreviations: NAA, α -Naphthalene acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid

tissues which are not involved in the production of plant through natural breeding. It can be induced from callus, cell suspension or protoplasts or directly from organized structures such as zygotic embryos and stem segments (Ammirato, 1983). After the first report on somatic embryos induction in carrot (Reinert, 1959), an increasing number of plant species have been cultured *in vitro* to induce somatic embryogenesis and to date, this phenomenon has been documented in more than 200 plant species (Kiyosue et al., 1993; Raemakers et al., 1995). Somatic embryogenesis is a direct result of interaction of heredity, developmental and culture environment regimes (Williams and Maheshwaran, 1986). Somatic embryogenesis can be induced directly from the explants or indirectly through the induction of embryogenic callus and differentiation of embryogenic cells through suspension cultures. Suspension cultures are generally initiated from friable callus and during the regeneration phase, somatic embryogenesis is easily observed (Kanwar and Kumar, 2008). There are distinct differences between embryogenic and non-embryogenic cells (Verdus et al., 1993; Yeung, 1995). Ultrastructurally, embryogenic cells have dense cytoplasm with numerous free ribosomes, mitochondria, plastids with starch grains (McCain et al., 1988; Fransz and Schel, 1991). The conversion of somatic embryos into plants is very critical and is attained through maturation and germination stages.

To date, there is no major report on the induction of somatic embryogenesis in *G. jamesonii*. Thus, the aim of the present study was to establish somatic embryogenesis and plant regeneration of *G. jamesonii* through suspension cultures. This research output could be exploited to select useful traits at the cellular level and to obtain improved plants or genetically stable cells.

MATERIALS AND METHODS

All experiments were conducted at B2.5 Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur Malaysia in year 2009.

Explant preparation

Seeds of *G. jamesonii* were surface sterilized in 40% commercial bleach (Clorox) for 20 min, followed by soaking in 70% alcohol for 1 min and finally by three rinses for 15 min each with sterile distilled water. Seeds were germinated aseptically on MS (Murashige and Skoog, 1962) basal medium fortified with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The pH of MS medium was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min.

Callus induction

For embryogenic callus induction, leaf explants taken or isolated from the aseptic seedling were used as source of explants and cultured on MS medium with 3% (w/v) sucrose and 0.8% (w/v) agar supplemented with various concentrations of 2,4-D. Cream friable

callus was induced after 2 weeks and subculturing was performed at 2 weeks intervals. Cultures were incubated in the culture room at 25 ± 2°C, 16 h light/8 h dark photoperiod, illuminated with white fluorescent light (30 μmol m⁻²S⁻¹). Twenty eight-day-old callus was used to establish cell suspension cultures.

Cell suspension culture

For initiation of cell suspension cultures, 28-day-old cream friable callus were transferred to 125 ml Erlenmeyer flasks containing 50 ml MS liquid medium supplemented with various concentrations of 2,4-D together with 0.1 or 1.0 mg/L NAA and 3% sucrose. Subsequently, the same set of culture medium was prepared and 5.0 mg/L L-glutamine or L-proline was added to the culture medium, respectively, in order to investigate the effect of this amino acid on the formation of somatic embryos in the suspension medium. The suspension cultures were incubated in the dark on a rotary shaker at 120 rpm, 25 ± 2°C. Cell suspension was subcultured every 7 days by replacing 20 ml of the old suspension medium with 20 ml of fresh medium. At the end of the third subculture, cell clusters were developed into globular and heart shaped somatic embryos. Embryogenic cells structures from the suspension were observed microscopically during the culture period. For maturation, the somatic embryos were later transferred to fresh MS basal medium together with 30% (w/v) sucrose and 0.8% (w/v) agar.

Plant regeneration

Mature cotyledonary stage embryos converted into plantlets after 21 days of culture when transferred to MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA together with 3% (w/v) sucrose and 0.8% (w/v) agar.

Scanning electron microscopy

Somatic embryos stages isolated from the cell suspension cultures were observed under scanning electron microscope to distinguish different stages of the somatic embryos formed.

Statistical analysis

30 replicates were used in each treatment and repeated thrice. Experimental design was completely random and factorial with callus initiation, globular, heart, torpedo and cotyledonary stages of the callus. All data were subjected to analysis of variance and mean separation was carried out using Duncan's multiple range test (DMRT) and significance of differences was determined at 5% level.

RESULTS AND DISCUSSION

Callus induction

In the present study, vigorous cream friable callus proliferation was observed after 14 days of culture (Table 1). Optimum embryogenic callus induction (100%) was observed on MS medium supplemented with 1.8 to 2.0 mg/L 2,4-D. Meanwhile, the lowest embryogenic callus induction was detected on MS medium fortified with 0.1 mg/L (38.3 ± 1.0%). Ammirato (1984) reported that 2,4-D has proven to be useful in obtaining somatic embryo-

Table 1. *In vitro* embryogenic callus induction from leaf explants of *G. jamesonii* cultured on MS medium supplemented with 2,4-D after 2 weeks.

2,4-D (mg/L)	Embryogenic callus frequency (%) Mean \pm SE
0.1	38.3 \pm 1.0 ^g
0.5	45.2 \pm 0.5 ^f
1.0	60.5 \pm 1.8 ^e
1.1	63.8 \pm 1.3 ^e
1.2	65.0 \pm 2.0 ^e
1.3	72.1 \pm 1.5 ^d
1.4	76.0 \pm 0.6 ^c
1.5	88.7 \pm 1.1 ^{bc}
1.6	95.3 \pm 1.0 ^b
1.7	98.7 \pm 0.4 ^b
1.8	100 ^a
1.9	100 ^a
2.0	100 ^a

Values are mean of 30 replicates per treatment and repeated thrice. Values of the same letter in the same column did not differ significantly at $p = 0.05$ level according to DMRT.

genesis response in a number of plant species. In addition, even low concentrations of 2,4-D influenced the induction of somatic embryogenesis when added to the culture medium (Martin, 2004). Similar data was observed in the present study. Luo et al. (1999) reported that embryogenic callus of *Astragalus adsurgens* Pall, a type of legume was induced when the culture medium was fortified with NAA and BAP. Meanwhile, similar results were reported in some *Lilium* species (Tribulato et al., 1997) and *Hordeum vulgare* L. (Sahrawat and Chand, 2004). Girija et al. (2000) described the induction of embryogenic callus from immature cotyledon explants of *Vigna radiata* on MS medium supplemented with 5.0 mg/L NAA. 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). Leaf explants were used as source of explants in *G. jamesonii* cv. Fredaisy for the initiation of non-embryogenic callus (Palai et al., 1998). Meanwhile Prasanth and Sekar (2004) reported that induction of callus from *G. jamesonii* cv. Mammut was achieved when leaf bits were cultured on medium containing 0.1 mg/L BAP and 0 to 3.0 mg/L NAA. Callus induction was also observed when leaf and petal explants of *G. jamesonii* were cultured on MS medium supplemented with 1.0 to 2.0 mg/L 2,4-D (Kumar and Kanwar, 2006). Ruffoni and Massabo (1991) reported that leaf laminae were capable of inducing callus of *G. jamesonii*. While Parthasarathy et al. (1996) obtained optimum callus growth of *G. jamesonii* when leaf explants were cultured on MS medium fortified with 1.0 mg/L NAA, BAP and IBA. Nonetheless, Orlikowska et al. (1999) reported that callus of *G. jamesonii* cv. Bal, Mariola and Rebecca were induced from petiole explants of the

youngest 3 to 4 leaves detached from axillary shoots produced *in vitro* when cultured on medium containing TDZ and NAA. The study reveals that the formation of embryogenic callus from cultured leaf explants of *G. jamesonii* was mainly dependant on the exogenous application of 2,4-D. In this study, 28-day-old cream friable callus were used to establish cell suspension culture to further induce stages of somatic embryogenesis in *G. jamesonii*.

Cell suspension culture

The embryogenic callus of *G. jamesonii* induced on MS solid medium supplemented with 2,4-D (1.8 to 2.0 mg/L) was transferred to MS liquid medium supplemented with the combination of 2,4-D and NAA with or without the addition of L-glutamine and L-proline. Dispersed callus cells (Figure 1A) started embryogenic stages that occurred as morphologically distinct cells, that is, rounded, elongated, spiral, etc. Rounded and elongated cells were enriched with cytoplasm within 7 days of culture. Attempt has been made to develop the early ontogeny stages of somatic embryos differentiation from liquid cultures of legumes. The approach of the present study was similar to previously reported procedures in which there was somatic embryo development through a division of spherical, spiral or elongated cells (Figure 1B). Eventually, the denser cytoplasmic cells were sustained and capable of organizing into multicellular aggregates that were considered to be proembryogenic mass. These proembryogenic mass later developed into globular (Figure 1C), heart (Figure 1D), torpedo (Figure 1E) and cotyledonary (Figure 1F) embryos within 2 weeks. The optimum frequency of somatic embryo formation was

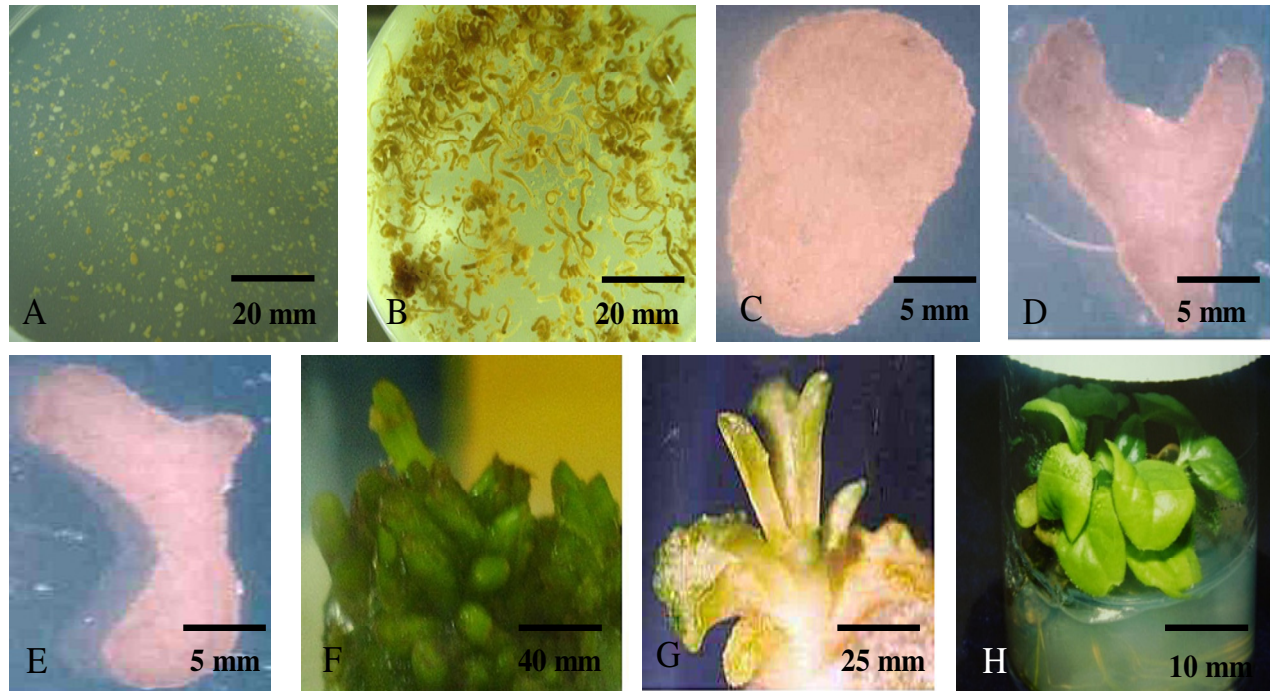


Figure 1. Somatic embryogenesis of *G. jamesonii* from suspension culture. (A) Dispersed callus cells; (B) distinct cells of somatic embryos (rounded, spiral and elongated from suspension culture); (C) globular embryo; (D) heart-shaped somatic embryo; (E) torpedo-shaped somatic embryo; (F) cotyledonary phase somatic embryo; (G) microshoots emerged from somatic embryo; (H) plantlets derived from somatic embryos.

achieved in MS liquid medium supplemented with 1.0 mg/L 2,4-D and 0.1 mg/L NAA together with 5.0 mg/L L-proline. Different stages of somatic embryos, globular ($91.3 \pm 1.0\%$) (Figure 1C), heart ($81.2 \pm 1.2\%$) (Figure 1D), torpedo ($59.6 \pm 0.5\%$) (Figure 1E) and cotyledonary ($48.3 \pm 1.5\%$) (Figure 1F) were observed (Table 2). The supplementation of L-glutamine or L-proline at 5.0 mg/L, respectively to the liquid culture media was proven to be effective in yielding high response of embryogenic frequency (Table 2). Nevertheless, somatic embryo stages failed to form when embryogenic callus were transferred to MS liquid medium supplemented with 1.0 or 2.0 mg/L 2,4-D alone. Moghaddam and Taha (2005) reported that the addition of L-proline to the culture medium enhanced somatic embryogenesis frequency in sugar beet. Finer and Nagasawa (1988) and Tetu et al. (1990) documented the beneficial effects of L-glutamine in soybean suspension cultures. Girija et al. (2000) reported that medium supplemented with L-proline was found to be beneficial for somatic embryogenesis in soybean and green gram. These results are in support of the present study where L-glutamine and L-proline were effective in somatic embryo induction of *G. jamesonii*. Sharma and Srivastava (2005) reported that somatic embryogenesis of *G. jamesonii* cv. Alsmeara was achieved when callus derived from leaf disc explants was cultured on suspension medium supplemented with 0.5 mg/L BAP and 1.0 mg/L NAA. Meanwhile, plant

regeneration of *G. jamesonii* cv. Diablo was observed from callus and suspension cultures derived from disc floret, ray floret and petal explants. Awal et al. (2008) reported that somatic embryogenesis of *Begonia x hiemalis* Fotsch was achieved on MS medium supplemented with 1.0 mg/L BAP, 0.1 mg/L 2,4-D and the addition of 100 mg/L casein hydrolysate. Somatic embryogenesis of *Gymnema sylvestre* was induced when 5.0 to 20.0 mg/L L-glutamine was added to MS liquid medium containing 0.5 mg/L 2,4-D or 1.0 mg/L NAA with the addition of 0.5 to 1.5 mg/L BAP (Ahmad et al., 2009). Induction of somatic embryogenesis from cell suspension cultures of *Astragalus chrysochlorus* was highly achieved when 3.0 mg/L IAA was supplemented in the culture medium (Turgut-Kara and Ari, 2008). Consequently, in inducing somatic embryogenesis, response of explants to auxin varies significantly depending on the plant species and the type of auxin used (Lakshmanan and Taji, 2000). Mature cotyledonary somatic embryos produced shoots (Figure 1G) and roots when transferred to MS solid medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA. This MS solid medium greatly enhanced the embryo germination and effectively stimulated plantlet development from somatic embryos of *G. jamesonii* (Figure 1H). In this present study, the low frequency of cotyledonary stage embryos of *G. jamesonii* might be as a result of precocious root development which could inhibit the development of the shoot pole. Inhibition of

Table 2. Influence of growth regulators on differentiation of somatic embryos derived from embryogenic callus induced from leaf explant of *G. Jamesonii*.

Plant growth regulator (mg/L)				Different stages of somatic embryos (%)			
2,4-D	NAA	L-Glutamine	L-Proline	Globular	Heart	Torpedo	Cotyledonary
1.0	-	-	-	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a
1.0	0.1	-	-	77.5 ± 0.5 ^d	61.2 ± 0.6 ^d	38.9 ± 1.2 ^{cd}	26.0 ± 0.5 ^c
1.0	0.1	5.0	-	89.7 ± 0.8 ^b	76.0 ± 1.0 ^b	52.0 ± 1.0 ^b	34.8 ± 1.0 ^b
1.0	0.1	-	5.0	91.3 ± 1.0 ^b	81.2 ± 1.2 ^b	59.6 ± 0.5 ^b	48.3 ± 1.5 ^b
1.0	1.0	-	-	56.4 ± 1.1 ^f	48.5 ± 2.2 ^e	25.3 ± 0.6 ^e	14.4 ± 0.8 ^d
1.0	1.0	5.0	-	73.9 ± 1.4 ^e	65.6 ± 1.4 ^d	42.0 ± 2.1 ^c	30.7 ± 0.4 ^b
1.0	1.0	-	5.0	82.6 ± 0.6 ^c	74.7 ± 1.5 ^c	50.7 ± 2.5 ^b	33.1 ± 0.1 ^b
2.0	-	-	-	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a
2.0	0.1	-	-	62.7 ± 2.0 ^{ef}	50.4 ± 0.8 ^e	27.1 ± 1.0 ^e	12.1 ± 1.5 ^{de}
2.0	0.1	5.0	-	82.2 ± 1.5 ^{cd}	75.9 ± 1.1 ^c	52.7 ± 0.4 ^b	29.4 ± 0.7 ^{bc}
2.0	0.1	-	5.0	88.0 ± 0.5 ^c	77.3 ± 0.7 ^b	54.5 ± 0.5 ^b	31.5 ± 1.1 ^b
2.0	1.0	-	-	48.5 ± 0.3	39.1 ± 1.5 ^f	15.8 ± 0.1 ^f	7.3 ± 0.5 ^e
2.0	1.0	5.0	-	69.7 ± 0.8 ^e	57.8 ± 0.5 ^{de}	34.0 ± 0.5 ^d	19.0 ± 1.0 ^d
2.0	1.0	-	5.0	71.7 ± 1.0 ^{de}	64.0 ± 1.8 ^d	40.2 ± 0.2 ^c	25.9 ± 1.3 ^c

Values are mean of 30 replicates per treatment and repeated thrice. Values of the same letter in the same column did not differ significantly at $p = 0.05$ level according to DMRT.

shoot pole development by early root formation is a problem encountered in several plant species (Tournand et al., 1983; Tejavathi et al., 2000). 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). Very few attempts were made to regenerate *Gerbera* through somatic embryogenesis (Kanwar and Kumar, 2008).

Embryo germination and plant regeneration

The mature cotyledonary embryos, upon transfer into MS solid medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA together with 3% (w/v) sucrose and 0.8% (w/v) agar were used for germination (Figure 1G). Ahmed et al. (2009) reported that mature embryos germination of *G. sylvestre* was achieved when transferred to MS basal medium with B5 vitamins, 3% (w/v) sucrose and 0.8% (w/v) agar. In *Begonia x hiemalis*, the conversion of somatic embryos into plantlets was observed with the withdrawal of BAP and 2,4-D from the culture medium (Awal et al., 2008). Germinated plantlets of *G. jamesonii* (Figure 1H) were individually transferred to garden soil and later acclimatized in the green house. About 11% of somatic embryos converted into true-to-type fertile plants. Acclimatized plants showed normal growth with similar phenotypic characters to the mother plants. The present

study indicates that although the formation of cotyledonary somatic embryos of *G. jamesonii* was low, nonetheless, the micropropagation capacity of the shoots derived from somatic embryos was high.

Scanning electron microscopy

Various somatic embryo stages of *G. jamesonii* were observed under scanning electron microscope. Globular embryos (Figure 2A) differentiated into heart (Figure 2B) and torpedo (Figure 2C) shaped embryos which later developed into cotyledonary (Figure 2D) stage embryos. Microscopic observations confirmed the ontogeny of somatic embryo development in *G. jamesonii*.

Conclusion

In conclusion, somatic embryogenesis of *G. jamesonii* was induced from leaf explants derived embryogenic callus in suspension culture. Embryogenic callus induction was optimal in MS solid medium supplemented with 1.8 to 2.0 mg/L 2,4-D. Somatic embryos frequency were highly achieved when embryogenic explants were transferred to MS liquid medium supplemented with 1.0 mg/L 2,4-D and 0.1 mg/L together with 5.0 mg/L L-glutamine. This efficient somatic embryos induction protocol could be useful for development, improvement and conservation of *G. jamesonii* in the horticulture

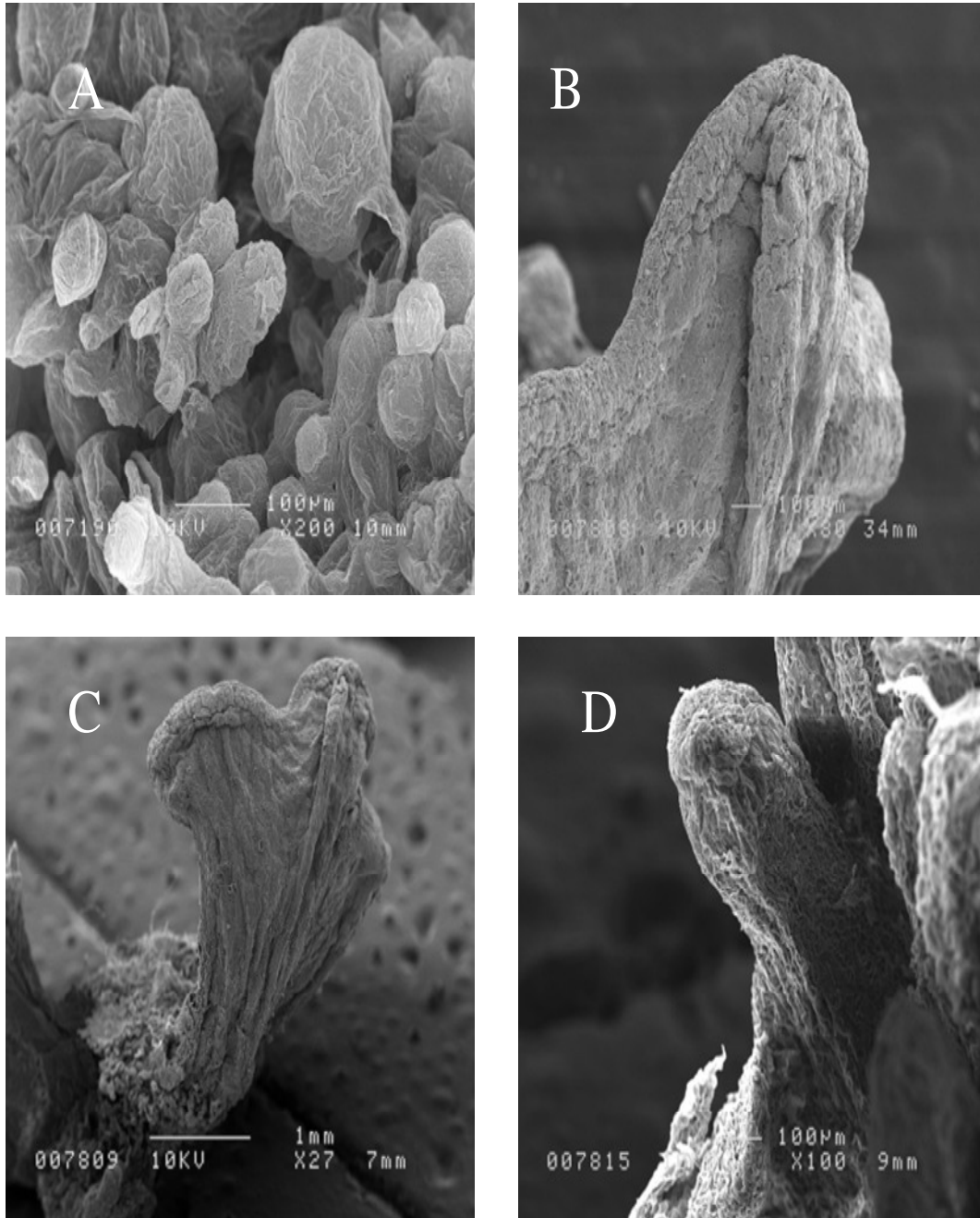


Figure 2. Scanning electron microscopy of globular-shaped somatic embryos. (A) globular- shaped; (B) heart-shaped (C) torpedo-shaped; (D) cotyledonary phases.

industry.

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