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Somatic embryo formation and germination from immature embryo-derived suspension-cultured cells of *Angelica sinensis* (Oliv.) Diels

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Abstract Embryogenic callus was induced from immature embryos of *Angelica sinensis* cultured on Murashige and Skoog (MS) basal medium. Embryogenic callus growth was more rapid on MS basal medium than on B5 or White medium. Embryogenic callus was used to establish a suspension culture and somatic embryos and germinating embryos developed during the culture. A shaking speed of 80 rpm was found to be optimal for establishing suspension cultures, while 100 rpm produced more somatic embryos and germinating embryos with an initiation cell density of 0.2 ml packed cell volume/25 ml medium. Adding 0.3% agar to the liquid medium also stimulated the formation of somatic and germinating embryos. While no plant growth regulators were needed for culture initiation and plant regeneration, the addition of 0.5–1 mg/l 2,4-dichlorophenoxyacetic acid was needed to maintain the embryogenic suspension culture by preventing embryo germination. Forty percent of the germinating embryos survived after culturing on filter paper moistened with liquid half-strength MS medium containing 3% sucrose. The plants were successfully transferred into soil.

Key words *Angelica sinensis* · Immature embryo culture · Somatic embryo · Suspension cell culture

Abbreviations *2,4-D* 2,4-Dichloroacetic acid · *MS* Murashige and Skoog · *PCV* Packed cell volume

Introduction

Dang Guei (*Angelica sinensis*) is a very valuable Chinese herb. It has been reported to enrich the blood, stimulate blood circulation, regulate menstruation, and relieve pain. Because of its broad pharmacological effects, it has been used for thousands of years in traditional Chinese medicinal prescriptions (Zhang and Cheng 1989). *A. sinensis* produces significant amounts of ferulic acid, ligustilide, butylphthalide, butylidenephthalide, allo-ocimene, and angelicide (Zhang and Cheng 1989). Studies on the pharmacology and clinical use of these active ingredients are becoming more extensive. The purpose of this study was to develop a propagation system for *A. sinensis* from immature embryos. The development of plant tissue culture methods will be vital to the extended utility of *A. sinensis* for pharmacological studies.

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Materials and methods

Flower buds of *A. sinensis* (Oliv.) with immature embryos (about 1–2 weeks after pollination) were collected from a field in Gan-Su Province in China. Buds were sterilized with 70% ethanol for 30 s, washed with 1% sodium hypochlorite solution for 5 min and finally rinsed five times with sterilized water. The immature embryos were removed aseptically from the ovary and cultured onto Murashige and Skoog (1962) (MS) basal medium. After 1 month, embryogenic callus induced from immature embryos was then subcultured onto MS, B5 (Gamborg et al. 1968) or White (1954) medium supplemented with 3% sucrose and 0.9% Difco agar to evaluate their growth ability. Each treatment contained a total of 54 embryogenic calli with each callus initial fresh weight of 20 mg. Callus fresh weight was determined after 30 days in culture. For establishment of rapidly growing and finely dispersed cell suspensions, embryogenic callus was cultured in a 125-ml Erlenmeyer flask containing 25 ml of MS liquid medium at 120 rpm shaking speed. The culture vessels were capped with two layers of aluminum foil and then wrapped with three layers of paraffin paper to avoid contamination. The cultures were maintained at 25±1 °C under a 16-h photoperiod (fluorescent light, 100 $\mu E/m^2$ per second for agar culture and 15 $\mu E/m^2$ per second for liquid culture). The suspension cells were passed through 0.59-mm-pore-size sieves after 30 days of culture. The established suspension cells were then used for testing culture conditions, e.g., shaking speed, initial cell density, media condition, embryo initiation, and plantlet formation. Cell growth was measured by packed cell volume (PCV) after 30 days of culture. The initial PCV of suspension cells in 125-ml De-long flasks containing 25 ml liquid basal medium was about 0.4 ml. Cell suspensions were maintained on a gyra-

tory shaker at 100 rpm for all experiments indicated unless otherwise. ANOVA and Duncan's multiple-range test were calculated for statistical analysis.

Results and discussion

Embryogenic calli and normal and abnormal plantlets were formed from immature embryos cultured on MS basal medium after 1 month of culture (Huang et al. 1996). The embryogenic callus was then subcultured into different basal media to evaluate growth ability. Embryogenic callus grew most rapidly on full- or half-strength MS basal medium among the four media tested (Table 1).

The effects of shaking speed, and initial PCV on establishment of cell suspensions are shown in Figs. 1 and 2. A shaking speed of 80 rpm was found to be superior to other speeds for the growth of cell suspensions (Fig. 1). The initial PCV significantly affected the growth rate of cell suspension cultures (Fig. 2). The stationary growth phase was reached within 10–12 days with an initial volume of 2.5 ml packed cells in 25 ml medium. However, a lower initial volume (0.2–0.4 ml PCV) was more suitable for long-term suspension cultures, as the stationary phase was delayed until 24 days after culture (Fig. 2).

Establishing a good culture system is a prerequisite for cell suspension culture research. Ammirato (1983) reported that during culture, suspension cells must be transferred to fresh medium periodically. The timing is especially critical as suspension cultures senesce rapidly at the end of the growth phase. Hsu et al. (1990) found that the duration of the logarithmic phase was dependent on initial cell concentration. With asparagus suspension cell culture, if the cells were initially cultured at low concentration, the logarithmic phase continued for a longer period. When cells were initially cultured at high concentrations, the logarithmic growth was shorter. The same tendency was found with the suspension-culture cells of *A. sinensis*. In addition, there are a number of studies demonstrating that frequent subculturing can effectively minimize the extent of chromosomal changes in cell cultures and maintain embryogenic potency (Bayliss 1977; Evans and Gamborg 1982). Therefore, in order to maintain *A. sinensis* suspension cultures that have active growth, high embryogenic potential, and low mutant generation, it is better to transfer the cells during the log phase of growth. We found that a medium containing MS basic salts and 3% sucrose was suitable for maintenance of the suspension cultures. The recommended shaking speed for establishing a cell suspension culture is 80 rpm. An initial PCV between 0.2–0.4 ml

Table 1 Influence of basal medium on the growth of embryogenic calli (*EC*) of *Angelica sinensis*. Basal medium: *MS* (Murashige and Skoog 1962), B5 (Gamborg et al. 1968) and White (1954) basic salts supplemented with 3% sucrose, 0.9% agar and pH=5.7. The initial fresh weight of each EC was 20 mg. The culture duration was 30 days. Means followed by the same letter are not significantly different at the 5% level using Duncan's multiple-range test

Basal medium	Number of EC cultured	Mean fresh weight (mg)
MS	54	236[a]
1/2 MS	54	249[a]
B5	54	148[b]
White	54	41[c]

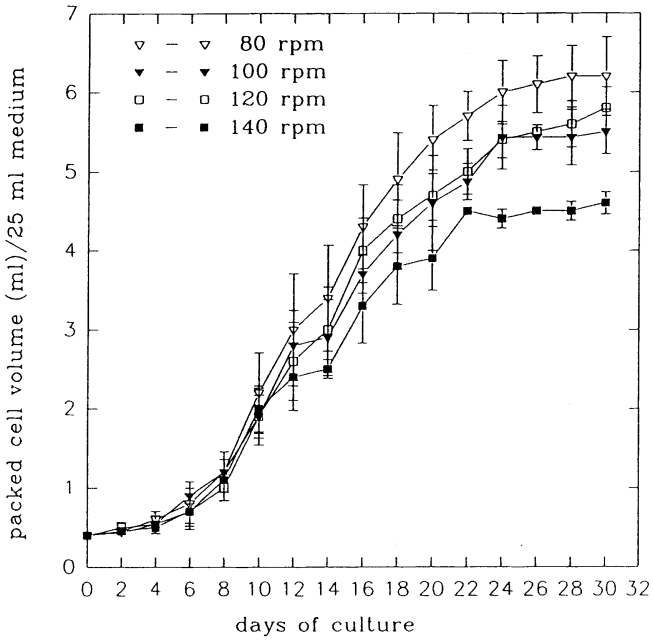

Fig. 1 Growth curve of suspension-cultured cells of *Angelica sinensis* cultured at different shaking speeds. Basal medium: MS salts supplemented with 3% sucrose

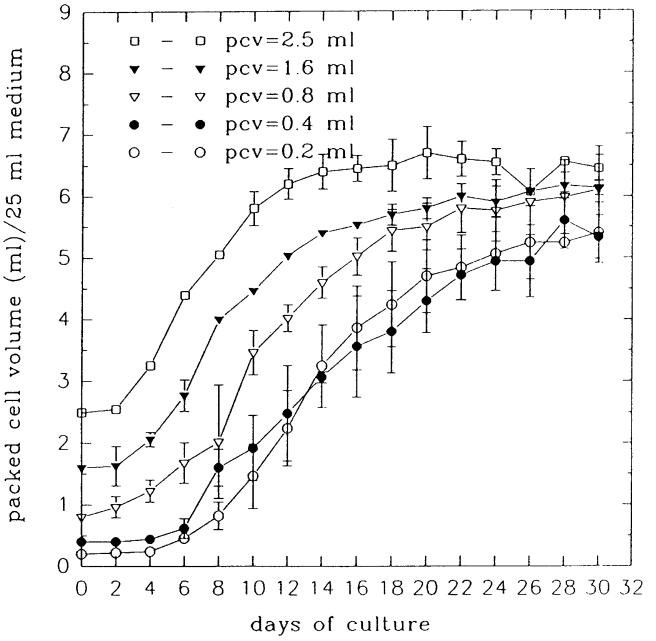

Fig. 2 Growth curve of suspension-cultured cells of *A. sinensis* cultured at different initial cell density and shaking at 100 rpm. Basal medium: MS salts supplemented with 3% sucrose

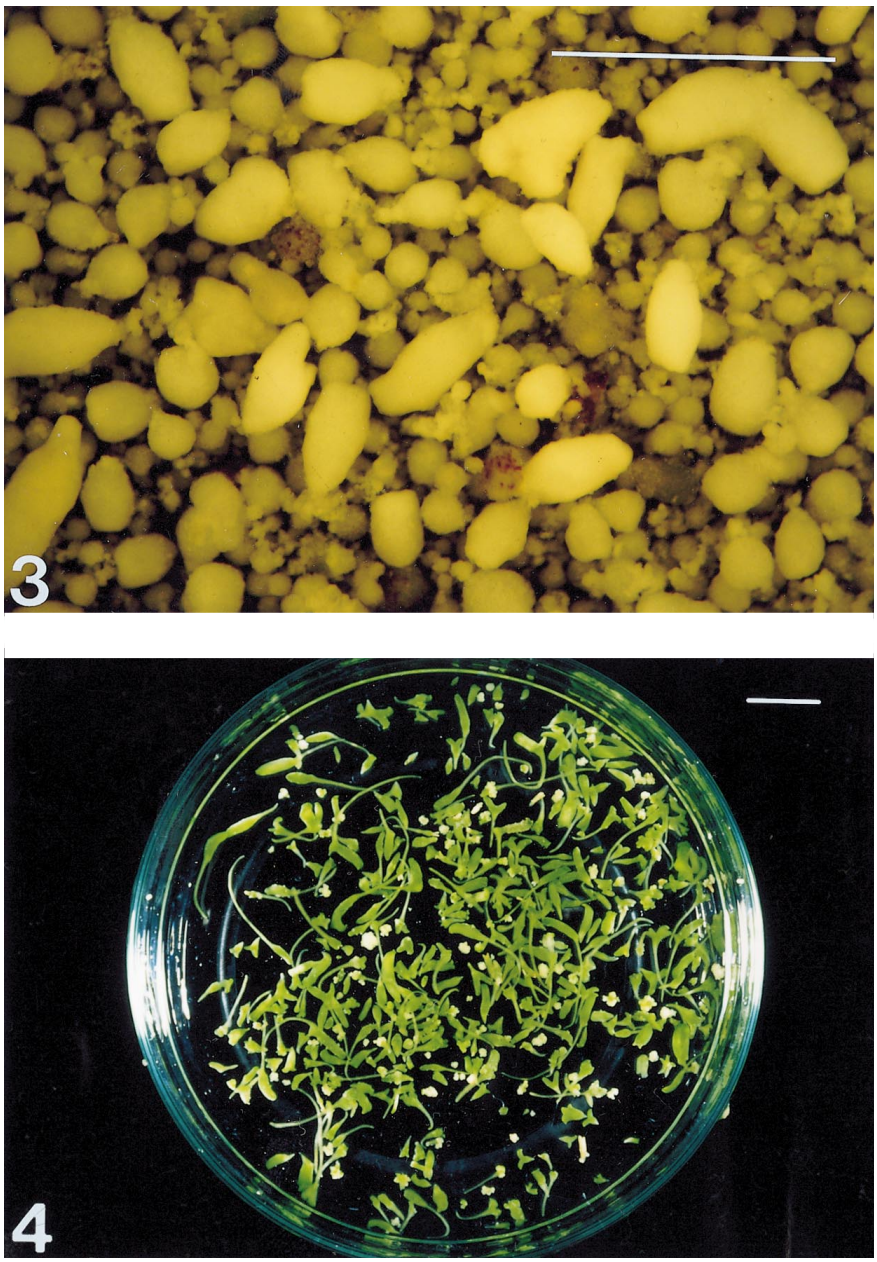

Fig. 3–4 Embryogenic callus was cultured in liquid MS medium, where it formed suspension cultures that developed further into somatic embryos (**Fig. 3**) and germinating embryos (**Fig. 4**) (*bar* 1 cm)

(per 25 ml liquid medium) was good for somatic embryo initiation and long-term suspension culture (Table 2). In this case, the cells must be cultured at 100 rpm and subcultured at 2- to 3-week intervals (Table 3).

The established-suspension cultured cells were grown in a liquid MS medium containing 0.5–1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in order to maintain their fine structure of cell clumps and to prevent formation of somatic and germinating embryos. When embryogenic callus was cultured in liquid MS medium (without 2,4-D), cells and cell clumps grew and developed into somatic embryos (Fig. 3) and germinated (Fig. 4). More somatic embryos and germinating embryos were formed when suspension cells were cultured at 100 rpm with a 0.2 ml initial PCV (Tables 2, 3). Adding 0.3% agar to the liquid medium at the beginning of the culture helped more somatic and germinating embryos form (Table 4). Forty percent of germinating embryos survived after culture on filter paper moistened with liquid 1/2 MS medium containing 3% sucrose (81 out of 202) (Fig. 5). These germinating embryos were successfully transferred into soil (Fig. 6) and grew for more than 6 months (Fig. 7).

Few papers describing the tissue culture of Dang Guei have been previously published, maybe because of its limited geographic distribution. Zhang and Cheng (1982, 1986, 1989) and Gu (1982) induced callus from root, leaf, petiole, cotyledon, and hypocotyl tissues. Plant regeneration was obtained from adventitious buds, somatic embryogenesis from callus differentiation. Auxins were reported to be beneficial for callus induction and differentiation oc-

Fig. 5 Germinating embryos on a filter paper moistened with liquid 1/2 MS medium containing 3% sucrose (*bar* 1 cm) (**Fig. 5**). These plants were successfully transferred into soil, (**Fig. 6**, pot diameter 20 cm) where they grew for 6 months (**Fig. 7**, pot diameter 11 cm)

Table 2 Influence of cell density on the formation of somatic embryos and germinating embryos in suspension-cultured cells of *A. sinensis*. Basal medium: MS basic salts supplemented with 3% sucrose, 0.9% agar and pH=5.7. Initial PCV of suspension cells in 125 ml Delong flasks containing 25 ml liquid medium was about 0.4 ml. Only somatic embryos larger than 1 mm in diameter and germinating embryos between 2–5 mm in length were counted. Each value is the mean of at least five replications. Means followed by the same letter are not significantly different at the 5% level using Duncan's multiple-range test. Shaking speed was 100 rpm

Initial PCV (ml)	Mean number of somatic embryos produced per flask	Mean number of germinating embryos produced per flask
0.2	344[a]	208[a]
0.4	148[a]	55[b]
1.0	108[b,c]	28[c]
1.6	75[c]	18[c]
2.5	63[c]	2[c]

Table 3 Influence of shaking speed on the formation of somatic embryos and germinating embryos in suspension-cultured cells of *A. sinensis*. Basal medium: MS basic salts supplemented with 3% sucrose, 0.9% agar and pH=5.7. Initial PCV of suspension cells in 125 ml Delong flasks containing 25 ml liquid medium was about 0.4 ml. Only somatic embryos larger than 1 mm in diameter and germinating embryos between 2–5 mm in length were counted. Each value is the mean of at least five replications. Means followed by the same letter are not significantly different at the 5% level using Duncan's multiple-range test

Shaking speed (rpm)	Mean number of somatic embryos produced per flask	Mean number of germinating embryos produced per flask
80	44[c]	21[b]
100	106[a]	48[a]
120	75[b]	11[b]
140	50[c]	11[b]

curred after subculture to a medium containing reduced levels of auxin. In our experiments, embryogenic callus could be induced from immature embryos cultured on a simple MS medium without any added plant growth regulators. Subculture of these immature embryo-derived calli onto the same medium resulted in embryogenesis and plant regeneration. The potential for continuous somatic embryo formation from our cultured cells has been retained on the MS medium with 3% sucrose for 8 years (unpublished data).

In conclusion, the culture systems established from this study will be valuable for both mass propagation and production of secondary metabolites from *A. sinensis*. Our current study shows that butylidene phthalide and ferulic acid, the two major components in *A. sinensis* can be detected by HPLC. Germinating embryos could be stored at 5 °C for months without losing their germination ability. These results strongly suggest that embryogenic callus derived from immature embryos of *A. sinensis* is excellent material for making artificial seeds and for studies of medicinal compound production.

Table 4 Influence of media composition on the formation of somatic embryos and germinating embryos in suspension-cultured cells of *A. sinensis*. For further details see legend to Table 3

Medium conditions	Mean number of somatic embryos produced per flask	Mean number of germinating embryos produced per flask
0.9% agar (solid)	0[c]	0[d]
0.1% gelrite (semi-solid)	355[b]	275[b]
0.3% agar (semi-solid)	565[a]	490[a]
No gelling agents (liquid)	230[b]	175[c]

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