Optimization of induction, subculture conditions, and growth kinetics of *Angelica sinensis* (Oliv.) Diels callus

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ABSTRACT

Background: *Angelica sinensis* (Oliv.) Diels is an important traditional Chinese medicine, and the medicinal position is its root. This perennial herb grows vigorously only in specific areas and the environment. Tissue culture induction of callus and plant regeneration is an important and effective way to obtain large scale cultures of *A. sinensis.* **Objective:** The objective was to optimize the inductive, subculture conditions, and growth kinetics of *A. sinensis*. **Materials and Methods:** Tissue culture conditions for *A. sinensis* were optimized using leaves and petioles (types I and II) as explants source. Murashige and Skoog (MS) and H media supplemented with 30 g/L sucrose, 7.5 g/L agar, and varying concentrations of plant growth regulators were used for callus induction. In addition, four different basal media supplemented with 1.0 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D), 0.2 mg/L 6-benzyladenine (BA) and 30 g/L sucrose were optimized for callus subculture. Finally, growth kinetics of *A. sinensis* cultured on different subculture media was investigated based on callus properties, including fresh weight, dry weight, medium pH, callus relative fresh weight growth, callus relative growth rate (CRGR), and sucrose content. **Results:** MS medium supplemented with 5 mg/L α-naphthaleneacetic acid, 0.5 mg/L BA, 0.7 mg/L 2,4-D, 30 g/L sucrose and 7.5 g/L agar resulted in optimal callus induction in *A. sinensis* while petiole I was found as the best plant organ for callus induction. The B_5 medium supplemented with 1.0 mg/L 2,4-D, 0.2 mg/L BA and 30 g/L sucrose displayed the best results in *A.* sinensis callus subculture assays. **Conclusion:** The optimized conditions could be one of the most potent methods for large-scale tissue culture of *A. sinensis*.

Key words: *Angelica sinensis,* callus induction, growth kinetics, plant organs

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INTRODUCTION

Angelica sinensis (Oliv.) Diels (Umbelliferae) is a perennial herb, which has been planted and used in China for 1000's of years, and it is also cultivated in Korea and Japan.[1] As an important traditional Chinese medicine, the dried root of *A. sinensis* mainly used to enriching blood and treat rheumatism, anemia, and menstrual problems.[2] In addition, *A. sinensis* has been widely used in the East because of its treatment of cardiovascular diseases.[3]

Thanks to its medicinal properties and use in health improvement, the demand for *A. sinensis* steadily increases. However, *A. sinensis* grows energetically only in specific areas and shows good medicinal properties only after 2 years of growth. In China, *A. sinensis* is well-known mainly in Gansu province, home to the most valuable type called Min Xian.[4] Traditional breeding presents many disadvantages: Long growth period, low efficiency, and quality and field production which tend to constantly decrease due to various diseases. Moreover, *A. sinensis* seeds must be sown on uncultivated soil, destroying natural vegetation.[5]

Tissue culture induction of callus and plant regeneration using various organs is an important and effective way to obtain large scale cultures of *A. sinensis.* Inducing multiple shoots from rhizomal buds has developed a propagation system for *A. sinensis*.[6] Zhang and Cheng[4,7,8] induced callus from roots, leaves, petioles, cotyledons, and hypercotyls, and regenerated plants by somatic embryogenesis and culture of adventitious buds from callus on five different media. Gu[9] had the similar results. Luo[5] used petioles, root tips, and laminas of seedlings as explants to investigate the effects of various concentrations of the hormone and different culture conditions on organic differentiation. They found a higher induction rate in calli obtained from

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petioles in comparison with that from root tips and laminas. However, few reports describing *A. sinensis* tissue culture are available, and the optimization of induction, subculture media, and growth kinetics has not been investigated in details.

In this study, *A. sinensis* from the South China Botanical Garden was used as explant in tissue culture system for the first time. Four different media were analyzed for optimization of callus induction in *A. sinensis*, using leaves and two different petiole types. Petiole I is the portion adjacent to the leaf whereas petiole II is adjacent to stem. In addition, four different basal media were evaluated for subculture optimization and growth kinetics of *A. sinensis* callus were investigated, in order to provide useful procedures to enrich the *in vitro* culture conditions.

MATERIALS AND METHODS

Preparation of tissue culture media

The explants were induced on four different media with properties summarized in Table 1. Calli were induced in a 100-mL Erlenmeyer flask containing 40 mL of liquid medium supplemented with sucrose and agar at 30 g/L and 7.5 g/L, respectively. The medium pH was adjusted to 5.75 before autoclaving for 20 min at 121°C.

Calli were subcultured in 250-mL Erlenmeyer flasks containing 80 mL defined media supplemented with 30 g/L sucrose (pH 5.75), autoclaved as described above. The properties of the callus subculture media are shown in Table 2.

Plant material

Leaves and two different petiole types were collected from *A. sinensis*, provided by The South China Botanical Garden, Guangzhou, China.

Callus induction assays

Explants were washed completely under running tap water for 30 min, surface-sterilized for 1 min by soaking in 75% (v/v) ethanol, overall-sterilized for 8–10 min in 0.1% (w/v) aqueous solution of mercuric chloride, and washed 5 times in sterilized water. Leaves and petioles were aseptically cut into pieces (1 cm × 1 cm) and segments (1.5 cm), respectively, and cultured for induction of primary calli in a growth room at 25°C ± 1°C in the dark. Callus color, type, and induction frequency were evaluated after 40 d of culture (without transfer). We calculated the induction rate of callus formation as the percentage of the number of explants generating callus in the total number of explants induced on callus induction medium.[10]

Table 1: Composition of media used for callus induction

Formula	Basal media	2,4-D	NAA	BA
1	MS	-	0.7	1.5
2	MS	0.7	5.0	0.5
3	H	0.5	-	-
4	MS	1.0	-	-

NAA: α-naphthaleneacetic acid; 2,4-D: 2,4-dichlorophenoxy acetic acid; MS: Murashige and Skoog; BA: 6-benzyladenine

Table 2: Composition of media used for callus subculture

Formula	Basal media	2,4-D	BA
1	MS	1	0.2
2	SH	1	0.2
3	B5	1	0.2
4	White	1	0.2

MS: Murashige and Skoog; BA: 6-benzyladenine; SH: Schenk and Hildebrandt; 2,4-D: 2,4-dichlorophenoxy acetic acid

Callus subculture

Initially, calli were cultured in conical flasks, kept in the dark at 25°C ± 1°C and subcultured every 3 weeks. After several subcultures on the optimized callus induction medium, vigorously growing and loose callus cultures were selected for growth characteristic assessment. One hundred and forty-four 250-mL Erlenmeyer flasks containing 80 mL sterilized basal medium with 1.0 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D), 0.2 mg/L 6-benzyladenine (BA), and 30 g/L sucrose were prepared for inoculation. Growth kinetics study comparing the four basal medium formulations were performed using 2 g of callus as initial inoculum per flask on a rotary shaker at 110 rpm.

Assessment of growth kinetics

To determine the effects of basal media on callus growth, callus fresh mass and medium pH were measured from three randomly selected cultures in each treatment group every three days; Based on fresh mass, callus relative fresh weight growth (CRFWG) and callus relative growth rate (CRGR) were calculated according to the following formula:[11-13]

$$CRFWG = ([W2 - W1]/W1) \quad (1)$$

$$CRGR = (\ln W2 - \ln W1)/\text{Number of days} \quad (2)$$

where W1 is the average fresh weight after 0 days of callus culture, and W2 the average fresh weight after 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33 days of callus culture.

Callus samples of known fresh mass were dried to constant weights in an oven set at 50°C for 72 h. In addition, the sugar contents of the four basal media were measured by phenol sulfuric acid method. For standard curve, aqueous sugar solutions were prepared in deionized water from a 200 μg/mL sucrose solution [Table 3].

Table 3: Sucrose solutions used for the establishment of the standard curve

Reagents	0	1	2	3	4	5	6	7	8
Sucrose solution (mL)	0.0	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.4
Deionized water (mL)	2.0	2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6
Sucrose content (μg)	0	20	40	80	120	160	200	240	280

To each mixture 1 mL, 9% aqueous phenol was added followed by concentrated sulfuric acid (5 mL). After mixing (vortex) and cooling for 30 min, absorbance of the yellow product was read at 485 nm against a reagent blank. All analyses were performed in triplicate. The resulting standard curve was used to assess sugar contents in the samples.

RESULTS

Callus induction

Murashige and Skoog (MS) medium[14] containing 0.7 mg/L 2,4-D, 5 mg/L α-naphthaleneacetic acid (NAA) and 0.5 mg/L BA was the best medium for callus induction, displaying an average induction frequency of 61.7%. The effects of culture media on callus induction are shown in Table 4. Statistical analyses indicated that medium 2 yielded the highest frequency of callus induction. In contrast, medium 4 presented a remarkably low callus induction frequency and growth rates in comparison with the other media.

The effects of different organs on callus induction are summarized in Table 5. Statistical analyses demonstrated that petiole I was the best explants source for callus induction, compared with petiole II and leaf. The optimum media for *A. sinensis* leaf, petiole I, petiole II callus induction were media 3, 2, and 2, respectively.

Calli were formed from different organs (leaf, petiole I, and II) on the four media to evaluate growth ability. As shown in Figures 1-3, explants induced on medium 1 displayed the fastest growth rate, as evaluated by callus mass and color after 40 d of culture.

Leaves showed the highest callus induction frequency (40%) on H medium[15] containing 0.5 mg/L 2,4-D, 30 g/L sucrose and 0.75% (7.5 g/L) agar. Meanwhile, petiole I displayed highest callus induction frequency (92.5%) on MS medium supplemented with 5 mg/L NAA, 0.5 mg/L BA, 0.7 mg/L 2,4-D, 30 g/L sucrose and 0.75% (7.5 g/L) agar, whereas petiole II showed highest callus induction frequency (70%) on MS medium supplemented with 5 mg/L NAA, 0.5 mg/L BA, 0.7 mg/L 2,4-D, 30 g/L sucrose, and 0.75% (7.5 g/L) agar. However, calli induced on medium 1 displayed the fastest growth rates, as evaluated by callus mass and type after 40 d of culture.

Table 4: Effects of induction culture media on callus formation

Media	Explants	Calli induced	Inoculum amount	Induction frequency (%)	Average (%)
1	Leaf	12	40	30	9.2
	Petiole I	34	40	85	
	Petiole II	25	40	63.5	
2	Leaf	9	40	22.5	61.7
	Petiole I	37	40	92.5	
	Petiole II	28	40	70	
3	Leaf	16	40	40	35
	Petiole I	11	40	27.5	
	Petiole II	15	40	37.5	
4	Leaf	4	40	10	6.7
	Petiole I	1	40	2.5	
	Petiole II	3	40	7.5	

Table 5: Effects of different organs on callus induction

Explants	Media	Calli induced	Inoculum amount	Induction frequency (%)	Average (%)
Leaf	1	12	40	30	25.6
	2	9	40	22.5	
	3	16	40	40	
	4	4	40	10	
Petiole I	1	34	40	85	51.9
	2	37	40	92.5	
	3	11	40	27.5	
	4	1	40	2.5	
Petiole II	1	25	40	62.5	44.4
	2	28	40	70	
	3	15	40	37.5	
	4	3	40	7.5	

Pale-yellow and green calli were subcultured while red/brown calli were discarded for avoiding it led to calli death.[16] In plant tissue culture experiments, evaluation of callus fresh or dry weight after a decided time interval usually as a measurement of the growth of callus.[17] Figure 4 shows growth kinetics parameters of *A. sinensis* calli cultured on four different media. Both B_5[18] and SH (Schenk and Hildebrandt)[19] media displayed superior growth kinetics parameters for *A. sinensis* calli compared with MS and White media[20] [Figure 4a-e].

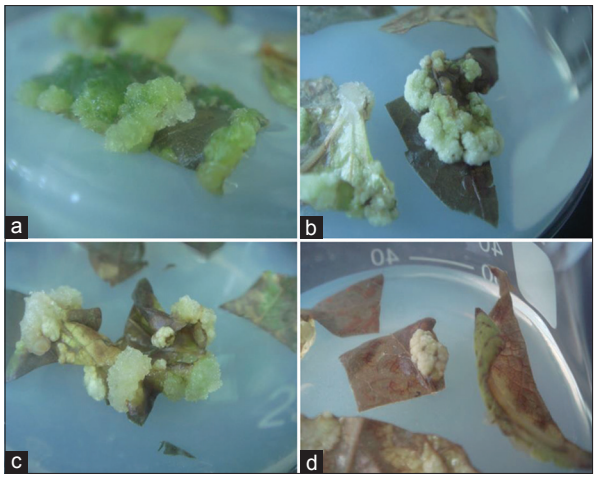

Figure 1: Callus induction from *Angelica sinensis* leaf (a) medium 1; (b) medium 2; (c) medium 3; (d) medium 4

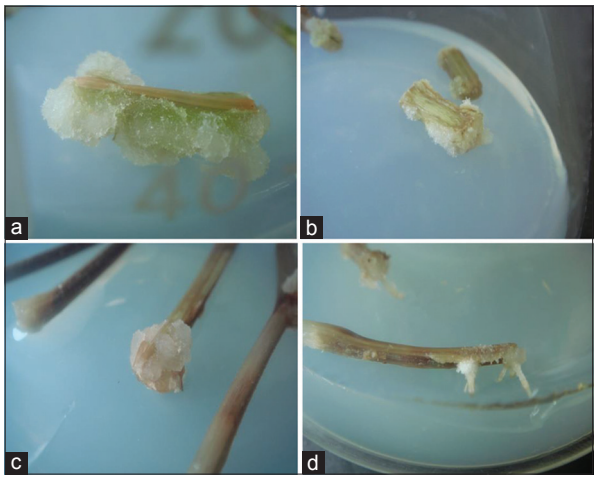

Figure 2: Callus induction from *Angelica sinensis* petiole Ⅰ (a) medium 1; (b) medium 2; (c) medium 3; (d) medium 4

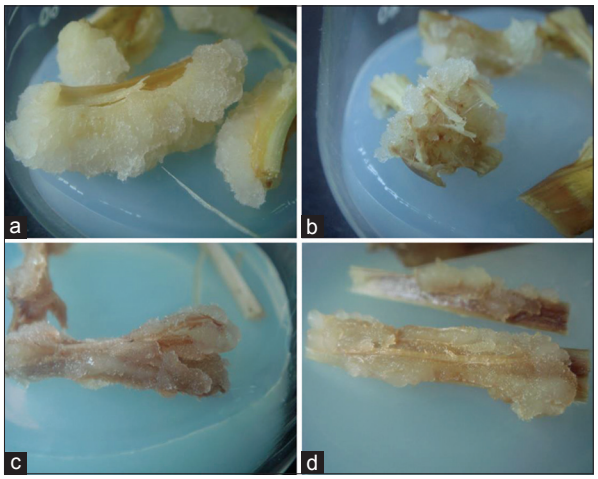

Figure 3: Callus induction from *Angelica sinensis* petiole Ⅱ (a) medium 1; (b) medium 2; (c) medium 3; (d) medium 4

The B_5 medium was selected over SH for *A. sinensis* callus, although they both achieved similar maximum. The major difference between these two formulations lies on doubling times. Indeed, average callus doubling times were 6 and 9 days on B_5 and SH media, respectively. The doubling times were calculated based on fresh and dry weight data. Fresh weight increase is well-correlated with an increase in dry weight. On MS medium, callus growth was reduced, reaching only half and quarter of that observed with White and B_5 media, respectively. Therefore, the slowest increase was observed with the MS medium.

Sugar levels were measured by a procedure that uses phenol-sulfuric acid reagents to produce yellow/brown products after the reaction with reducing sugars or carbohydrates. When expressed in terms of sucrose content (μg), the relationship was linear from 0 to 360 μg sucrose with the regression equation: $Y = 0.0041x + 0.0068$ ($r^2 = 0.999$). Graphs describing sucrose consumption of *A. sinensis* callus are shown in Figure 4f for the four media studied.

DISCUSSION AND CONCLUSIONS

In the present study, petiole Ⅰ was the best explant source for callus induction, ahead of leaves and petiole II. Similar results were got by Luo.[5] The callus induction frequency of leaves was the worst, in agreement with the reports by Zhang.[21] Our data showed that MS medium supplemented with 5 mg/L NAA, 0.5 mg/L BA, 0.7 mg/L 2,4-D, 30 g/L sucrose, and 7.5 g/L agar was the best medium for callus induction. The optimal medium for callus subculture was B_5 basal medium supplemented with 1.0 mg/L 2,4-D, 0.2 mg/L BA and 30 g/L sucrose, results not in line with reports by Tsay and Huang.[22] They induced embryogenic callus from immature embryos of *A. sinensis*, and the results showed that embryogenic callus growth was more vigorous on MS basal medium than on White or B_5 medium.

CONCLUSION

Our results suggest the possibility to improve callus induction frequency by optimizing the composition of callus induction media, plant organs, and callus type. Further investigation is underway to examine plant regeneration from the calli induced in this work.

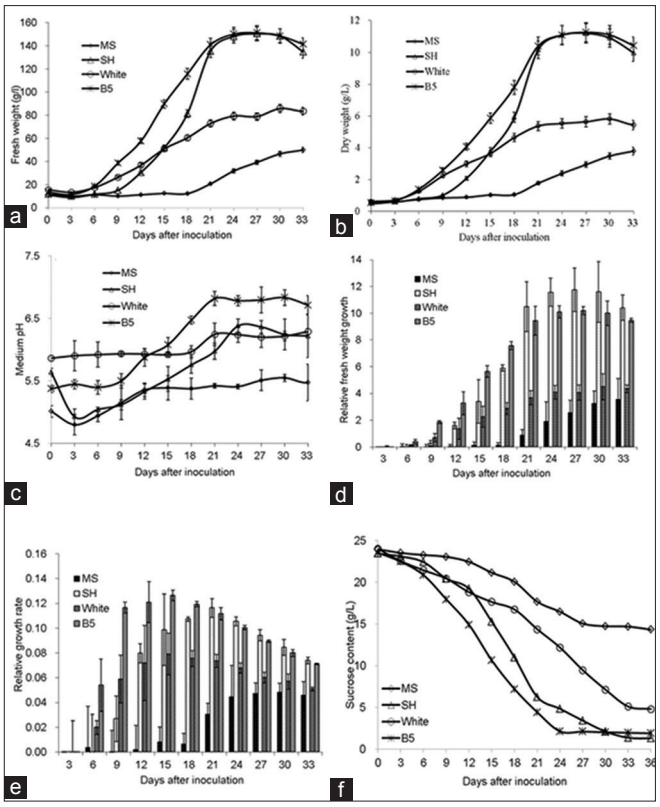

Figure 4: The growth kinetics of *Angelica sinensis* callus on four different media (a) fresh weight; (b) dry weight; (c) medium pH; (d) relative fresh weight growth; (e) relative growth rate; (f) sucrose content graphs obtained from the four basal media

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REFERENCES

1. Fang L, Xiao XF, Liu CX, He X. Recent advance in studies on *Angelica sinensis*. Chin Herb Med 2012;4:12-25.
2. Zhang HY, Bi WG, Yu Y, Liao WB. *Angelica sinensis* (Oliv.) Diels in China: Distribution, cultivation, utilization and variation. Genet Resour Crop Evol 2012;59:607-13.
3. Yeh JC, Cindrova-Davies T, Belleri M, Morbidelli L, Miller N, Cho CW, *et al.* The natural compound *n*-butylidenephthalide derived from the volatile oil of Radix *Angelica sinensis* inhibits angiogenesis *in vitro* and *in vivo*. Angiogenesis 2011;14:187-97.
4. Zhang SY, Cheng KC. *Angelica sinensis* (Oliv.) Diels *in vitro* culture regeneration, and the production of medicinal compounds. In: Bajaj YP, editor. Biotechnology in Agriculture and Forestry. Vol. 7. Medicinal and Aromatic Plants. Heidelberg, New York: Springer; 1989. p. 1-22.
5. Luo XF, Yang N, Chen XL, Ding L, Zhao QF, Ma RJ. Study on tissure culture of the plantlet germinated from seed of *Angelica sinensis* (Oliv) Diels. J Northwest Norm Univ 2004;40:77-9.
6. Mongkolchaipak T, Suchantaboot P. Studies on tissue culture of *Angelica sinensis* (Oliv.) Diels. J Thai Tradit Altern Med 2010;8:2-3.
7. Zhang SY, Cheng KC. Callus induction and plantlet regeneration from several organs of *Angelica sinensis* (Oliv.) Diels. Acta Bot Sin 1982;24:512-8.
8. Zhang SY, Cheng KC. Induction of embryogenic callus and histocytological study on embryoid development of *Angelica sinensis* (Oliv.) Diels. Acta Bot Sin 1986;28:241-4.
9. Gu JW. Studies on tissue culture of *Angelica sinesis* (Oliv) Diels (author's transl). Yao Xue Xue Bao 1982;17:131-8.
10. Sun YL, Hong SK. Effects of plant growth regulators and L-glutamic acid on shoot organogenesis in the halophyte *Leymus chinensis* (Trin.). Plant Cell Tissue Organ 2010;100:317-28.
11. Chen JY, Yue RQ, Xu HX, Chen XJ. Study on plant regeneration of wheat mature embryos under endosperm-supported culture. Agric Sci China 2006;5:572-8.
12. AL-Khayri JM, AL-Bahrany AM. Growth, water content, and proline accumulation in drought-stressed callus of date palm. Biol Plant 2004;48:105-8.
13. Farshadfar E, Esmaili SS, Rasoli V. Chromosomal localization of the genes controlling callus induction and *in vitro* drought tolerance criteria in wheat-barley disomic addition lines using mature embryo culture. Ann Biol Res 2012;3:1334-44.
14. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 1962;15:473-97.
15. Bourgin JP, Nitsch JP. Production of haploid *Nicotiana* from excised stamens. Ann Physiol Veg 1967;9:377-82.
16. Wickremesinhe ER, Arteca RN. *Taxus* callus cultures: Initiation, growth optimization, characterization and taxol production. Plant Cell Tissue Organ 1993;35:181-93.
17. Conner AJ, Meredith CP. An improved polyurethane support system for monitoring growth in plant cell cultures. Plant Cell Tissue Organ 1984;3:59-68.
18. Gamborg OL, Miller RA, Ojima K. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 1968;50:151-8.
19. Schenk RU, Hildebrandt AC. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 1972;50:199-204.
20. White PR. Nutrition of excised plant tissue and organs. Res Probl Biol Investig Stud 1963;2:203.
21. Zhang JL, Mi SE, Luan WJ. Analysis of callus induction conditions of *Angelica sinensis* (Oliv.) Diels. Gansu Agric Sci Technol 1995;11:8-10.
22. Tsay HS, Huang HL. Somatic embryo formation and germination from immature embryo-derived suspension-cultured cells of *Angelica sinensis* (Oliv.) Diels. Plant Cell Rep 1998;17:670-4.

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