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Propagation of *Angelica archangelica* plants in an air-sparged bioreactor from a novel embryogenic cell line, and their production of coumarins

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Abstract

A spontaneously embryogenic cell line of the coumarin producing angelica [*Angelica archangelica* (L.) subsp. *archangelica*] was established *via* callus formation from seedlings grown from sterilized seeds on semi-solid, hormone-free modified B5 medium. The cell line has retained its embryogenic capacity for 5 years. The highest coumarin production for the cell line after 3 weeks of cultivation was achieved in the medium containing 3.0 % sucrose. Jasmonic acid had no statistically significant effect on the biomass or coumarin production. The established embryogenic cell line could be stored using cryopreservation. Plantlets grown in an air-sparged bioreactor were transferred directly to soil and vermiculite, and 63 % of them grew to maturity through two growth seasons. The coumarin content in the regenerated plants was comparable to that in wild plants. Thus this cell line could be used for *in vitro* propagation.

Additional key words: somatic embryogenesis, regeneration, preservation, coumarin analysis.

Introduction

Angelica [*Angelica archangelica* (L.) subsp. *archangelica*, *Apiaceae*] is distributed throughout the Northern Europe and Eastern Siberia. The plant contains essential oils (Nykänen *et al.* 1991) and coumarins (Ojala 2001). Angelica is cultivated except in Finland, *e.g.*, in Hungary, France, the Netherlands, and Belgium, for its flavoring qualities in food and liqueur production.

Somatic embryogenesis provides a means of producing plant material, enabling not only developmental studies but also plant propagation. For the first time, somatic embryogenesis was reported in *Daucus carota* L. (Steward *et al.* 1958). Also other *Apiaceae* species have shown embryogenic activity: *Carum carvi, Petroselinum hortense* (Ammirato 1983), *Apium graveolens* (Nadel *et al.* 1989), *Ammi visnaga* (El Fiky *et al.* 1989), *Peucedanum palustre* (Vuorela *et al.* 1993), *Thapsia garcanica* (Jaeger *et al.* 1993), *Foeniculum vulgare* (Hunault and Mataar 1995), *Coriandrum sativum* (Kim *et al.* 1996), *Angelica sinensis* (Tsay and Huang 1998), and *Heracleum candicans* Wall (Wakhlu and Sharma 1998). According to Ekiert (2000) regenerative processes of 14 species of *Apiaceae* family have been investigated.

The germination ability of *A. archangelica* seeds was low (about 20 %) in a study conducted on Finnish and Swedish samples (Ojala 1985). Seeds stored at +5 °C lost their germinability after 40 months, and at room temperature after one year. Germination also required cold stratification as well as controlled light conditions. Further, the maintenance of cell cultures is often unpractical and may lead to loss of desired characteristics, *e.g.* ability to embryogenesis. Thus a preservation method such as cryopreservation offers a possibility for long-term storage of a plant cell line (Cho *et al.* 2000).

The objective of the present study was to establish an

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Abbreviation: JA - jasmonic acid.
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embryogenic cell line and to characterize its nutritional requirements. The effect of growth conditions on coumarin production was investigated. The culture conditions for a simple air-sparged bioreactor were optimized in order to enable large-scale plantlet propagation for field cultivation. Suitability of cryopreservation for storage of the established cell line was also studied.

Materials and methods

The embryogenic cell line of *Angelica archangelica* (L.) subsp. *archangelica* was established from ripe seeds originating from several umbels of a wild mother plant in Jukuanvaara, Finland (65°47′N, 27°32′E). The seeds were surface-sterilized with a solution of 0.01 % $HgCl_2$ and *Tween 80* for 15 min, and rinsed in sterilized water 3 times for 5 min. After peeling, the seeds were germinated in the dark on a medium containing 75.0 % of a hormone-free, modified B5 medium (Gamborg *et al.* 1968), 2.0 % (m/v) sucrose and solidified with 0.80 % (m/v) agar. The seeds grew into callus-forming seedlings. Embryo formation started spontaneously via subcultured callus, which was maintained using media containing 1.5 % (m/v) sucrose (called basal B5 medium with agar). The liquid cultures were carried out at 25 ± 2 °C under a 16-h photoperiod (irradiance 30 μmol m^{-2} s^{-1}) on a rotary shaker at 90 rpm in 100 cm^3 conical flasks containing 20 cm^3 basal B5 medium (without agar). All the experiments were made at least in triplicate.

During maintenance, the embryogenic stock cultures were subcultured every 3 weeks and synchronized every 6 weeks by sieving through successive mesh sizes (globular embryos mesh sizes 125 to 250 μm; heart-shaped and early torpedo-shaped embryos 250 to 500 μm; mainly torpedo-stage embryos 500 to 1000 μm). The cultures were microscoped frequently (*Nikon SMZ-10*, *Nikon Co*, Tokyo, Japan). For cryopreservation about 100 embryos (size < 250 μm) were transferred to 2-cm^3 cryotubes (*Nunc A/S*, Roskilde, Denmark) and 1.5 cm^3 of pre-chilled B5 medium containing 1 M glycerol was added. The tubes were kept at +4 °C for 1 h swirling them periodically and then the temperature was lowered gradually to -35 °C in 40 min. After another 40 min the tubes were inserted into liquid nitrogen.

The growth rate of the culture was measured by cultivating 100 ± 5 mg of stock culture per flask for 70 d. Samples were taken 3 times during the first week and then once every week.

In order to determine the optimum carbon source for the cell line sucrose, glucose, fructose, and xylose were tried. The concentration ranges in the experiments based on the monosaccharide unit equivalents of each sugar were 0.50 to 4.5 % (m/v) for sucrose, 1.1 to 6.3 % (m/v) for glucose and fructose, and 0.88 to 4.3 % (m/v) for xylose. The cultures were subcultured every 3 weeks on 3 occasions.

The effect of jasmonic acid (JA; *Sigma Chemicals Co*, St. Louis, USA) was studied by adding JA containing medium to culture flasks after 26 d of cultivation. The concentrations used were 0.1, 1, 10, 100, and 500 μM. The cultures were elicitated for 24, 48, 72, and 120 h. JA was dissolved in ethanol, diluted in a small volume of basal B5 medium, and filter-sterilized before use.

For production of plantlets, the embryogenic cell line was cultivated in an air-sparged, 5-dm^3 bioreactor (*Laborexin Oy*, Helsinki, Finland; working volume 3 dm^3, height 300 mm, ∅ 230 mm) for 28 d. The sterilized reactor was filled with medium and inoculated on a laminar flow bench with 10.0 g dm^{-3} 3-week-old stock culture. Forced aeration (3 dm^3 min^{-1}) was used in all the bioreactor experiments. Air was passed through a 0.1 % (m/v) $CuSO_4$ solution in order to minimize evaporation. The possibility of enhancing biomass production was studied using a fed-batch procedure in which 100 cm^3 of sterilized 60 % (m/v) sucrose solution was added twice a week to the reactor using a peristaltic pump. In the exponential growth phase of the culture, the developed plantlets were transplanted directly into a mixture of soil and vermiculite (30:70) from the reactor. For the first 3 weeks the plantlets were kept under glass at +18 °C. After that they could be transferred to soil and greenhouse conditions.

The plant material was separated from the medium by vacuum filtration, weighed and lyophilized for dry mass determination. The sucrose, glucose, and fructose concentrations of the media were measured using an enzyme-coupled colorimetric assay described by Hendrix (1993). For coumarin determinations by HPLC a 100-mg sample of lyophilized material was extracted with 3 cm^3 of methanol in a sonication bath for 30 min and centrifuged. An RP-HPLC system consisting of a *Waters 600E* system controller, multisolvent delivery system, *Waters 717* autosampler, photodiode array detector *991* (*Waters Corporation*, Milford, USA), and a *C-18 LiChroCART® 250-4 Hypersil ODS* (5 μm) column (*Merck KGaA*, Darmstadt, Germany) was used. Organic phase A contained ethanol (47.7 % v/v), acetonitrile (32.0 % v/v), tetrahydrofurane (4.8 % v/v) and 15.5 % (v/v) of phase B (water adjusted with H_3PO_4 to pH 3). The gradient was from 20 to 40 % of A in 13 min, from 40 to 80 % in 15 min, and 17 min isocratic with a flow rate of 1 cm^3 min^{-1}. The injection volume was 50 mm^3 and detection at 320 nm. *Bergapten* (*Fluka Chemie AG*, Buchs, Switzerland) was used as an external standard.

Identification of coumarins of the regenerated plants were based on standard compounds, retention times in HPLC, DAD-UV- and mass-spectra (data not shown here). For statistical analysis *Systat® 6.0.1. for Windows®* was used.

Results and discussion

The embryo formation capacity of the stock cultures of the established *A. archangelica* cell line has not decreased since its establishment 5 years ago. The stock culture maintained its viability after cryopreservation. No transfer onto any new intermediate induction or development media has been employed, which is often needed to achieve following developmental stages of embryogenesis or tissue formation (*e.g.* Laurain *et al.* 1996). Accumulation of coumarins was not detected in the isolated somatic embryo fractions (n = 4), but could be determined in the developed plantlets. In this case coumarin production started without exogenous auxins (*e.g.* Petit-Paly *et al.* 1999). The established *A. archangelica* culture grew slowly in the suspension culture. In the growth rate study, the highest dry mass (DM) and coumarin content of the cells were measured on day 56 (Fig. 1). The DM/FM ratio remained constant at 1/10 throughout the experiment. In corresponding studies, the maximum DM in *Peucedanum palustre* cultures were reached in four weeks (Vuorela *et al.* 1993), and in *A. sinensis* cultures already within 10 to 12 d (Tsay and Huang 1998). When all the sucrose had disappeared after day 28 (Fig. 1), there was a sudden jump in the coumarin content compared to the DM. After

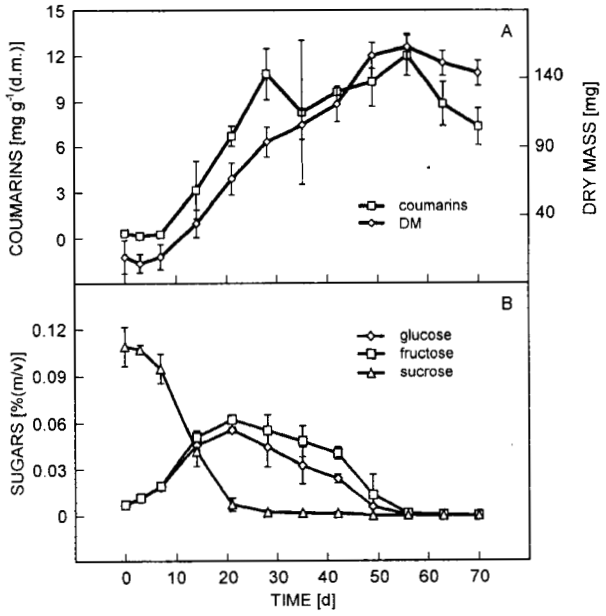

Fig. 1. Growth in suspension culture during 70 d of the embryogenic cell line of *A. archangelica* cultivated in 75 % basal B5 medium: *A* - dry mass and coumarin production, *B* - glucose, fructose or sucrose concentrations in the media.

Table 1. Effect of initial sucrose concentration on the measured amounts of sucrose, glucose and fructose in the growth media of the embryogenic cell line of *A. archangelica* after 21 d of cultivation (n = 3).

Initial	Sugars in medium after 21 d [%]					
sucrose [%]	glucose	SD [%]	fructose	SD [%]	sucrose	SD [%]
0.5	0.000		0.000		0.000	
1.5	0.000		0.000		0.000	
2.0	0.004	51.4	0.018	43.7	0.011	135.0
3.0	0.018	22.9	0.079	11.0	0.003	46.8
4.5	0.074	4.8	0.250	4.6	0.001	141.0

8 weeks all the sugars were utilized and the medium and plant material turned brownish and microscopic examination showed lysis of the cells.

In the carbon source experiments, only traces of sucrose were detected after cultivation, but the monosaccharides accumulated in the media if the initial sucrose concentration was higher than 2.0 % (Table 1). The DM in the sucrose-containing media varied between 132 ± 13 and 371 ± 21 mg flask^{-1}, reaching a maximum at 3.0 % sucrose. Coumarin production of the culture was highest (5.72 ± 0.15 mg g^{-1}) in the same medium. In *in vitro* studies of other plants in the *Apiaceae* family, callus cultures of *Pastinaca sativa* L. and *Ammi majus* L. were reported to produce up to 4.09 and 1.63 mg g^{-1} coumarins, respectively (Ekiert and Gomólka 2000, Ekiert 2000). We have shown earlier that more developed stages of embryos of *Peucedanum palustre* produced increased amounts of coumarins (up to 5.62 mg g^{-1}), being high (40.8 mg g^{-1}) and most diverse in the regenerated plant leaves (Härmälä *et al.* 1992). Although growth was similar in the glucose media (from 156 ± 11 to 296 ± 16 mg flask^{-1}) as in the sucrose media, the coumarin formation was clearly less in glucose media in comparison to sucrose media (coumarin content 1.59 ± 0.29 mg g^{-1} at 3.0 % glucose). When the initial sucrose concentration was increased (the content of glucose and fructose in media were high after 21 d of cultivation) the appearance of the cultures gradually changed from greenish plantlets to white embryogenic cell clusters. It has been recently published that the amount of carbon source also affected the number of nodes in hop mericlones (Smýkalová *et al.* 2001). On the other hand, when the media contained only traces of monosaccharides after cultivation the *A. archangelica* embryos regenerated readily into plantlets. Uozumi *et al.* (1991) have reported the possibility of using fructose as a sole

carbon source in fed-batch cultures of carrot hairy roots, however, in this case it inhibited embryo formation and the cultures stayed viable only at the lowest concentration (1.1 %). Also embryos cultured in xylose-containing media had no further development.

There were no statistically significant quantitative or qualitative effects of jasmonic acid (JA) on coumarin formation, but there was a clear tendency dependent on the incubation time and JA concentration. This has been reported also by Ellard-Ivey and Douglas (1996) and Miksch and Boland (1996) for other *Apiaceae* plant species. The coumarin content of the cultures was highest when incubated for 72 h with 10 μM JA (Fig. 2), and there were no coumarins detected in the media.

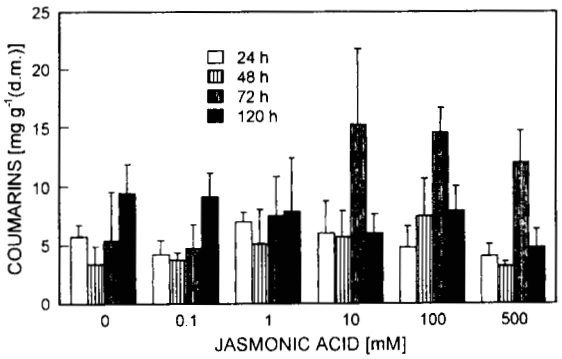

Fig. 2. Effect of different concentrations of jasmonic acid and incubation times on coumarin production of the embryogenic cell line of *A. archangelica*.

In the bioreactor batch cultivation the biomass production of the embryogenic *A. archangelica* culture was 2.3 g(FM) d^{-1}. Sucrose feeding did not enhance biomass production, but led to activation of embryogenesis. Thus plantlet formation was minimal and regeneration studies were therefore performed using plantlets from the bioreactor experiments without sucrose feeding. In this case plantlets were formed readily without any change of medium or addition of growth regulators. Shoots and roots were developing simultaneously, also at the same time new embryo formation was induced. Similar behaviour has been reported in our earlier work for *Peucedanum palustre* (Vuorela *et al.* 1993). After direct transfer from the bioreactor to unsterile conditions after 28 d of cultivation, 63 % of the 32 plantlets survived. The regenerated plants showed no morphological differences from normal plantlets as regards leaf, stem, or root formation. The coumarin content of the leaves of the regenerated 3-month-old plants (n = 9) varied between 1.95 and 8.06 mg g^{-1}, and was somewhat lower than in intact plants (max. 12.46 mg g^{-1}, data not shown) from the same area as the mother plant. Also seasonal changes in coumarin content of *Apiaceae* plants are known to be marked (Zobel and Brown 1990). The main coumarins in the regenerated leaves were isopimpinellin and isoimperatorin. Other compounds detected were bergapten, xanthotoxin, oxypeucedanin, osthrutol, and umbelliprenin. The coumarin composition of the leaves of the regenerated plants corresponded to that of intact plants (Ojala 2001). According to our results, the elevated embryogenic activity in fed-batch experiments enables the development of a two-phase cultivation system, in which cultivation is started with sucrose feeding in order to rapidly obtain a large amount of embryogenic material. Cultivation is then continued by replacing this medium with a low-concentration sucrose-medium in order to activate the regeneration of embryos into plantlets.

The production of artificial seeds is considered a promising tool for utilizing embryogenesis in plant production. However, sophisticated and expensive methods and equipment are often needed. In our study an alternative propagation method is presented. The stability of the established *A. archangelica* cell line, the good viability of the *A. archangelica* plantlets in soil propagated in a simple air-sparged bioreactor, and the simplicity of direct transfer into pots, make the developed method a promising means for the economical production of large amounts of *A. archangelica* plants for field cultivation.

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