Effects of combined hormonal deprivation and fungal elicitation on the production of coumarins in cell suspension cultures of *Angelica archangelica* L.

SIATKA T., KAŠPAROVÁ M.
Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Department of Pharmacognosy, Czech Republic

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SUMMARY

Effects of combined hormonal deprivation and fungal elicitation on the production of coumarins in cell suspension cultures of *Angelica archangelica* L.
The effectiveness of elicitation as a tool to enhance the production of secondary metabolites in plant tissue cultures depends on a complex interaction between the elicitor and the plant cell. The paper investigated the influence of removal of plant growth regulators and light conditions on the cell growth and elicitation of coumarins in *Angelica archangelica* cell suspension cultures. An autoclaved homogenate of *Pythium aphanidermatum* was used as the elicitor. Cell cultures of *Angelica archangelica* were cultured in a medium supplemented with 2,4-dichlorophenoxyacetic acid and benzylaminopurine or in a hormone free medium, and in the dark or under continuous light. The culture growth (both fresh and dry biomass) was not affected adversely in the presence of the fungal elicitor as compared with the control cultures. The contents of coumarins in cells were influenced slightly. The content of coumarins in the medium was stimulated by hormonal deprivation and elicitation depending on the light conditions; the best results were achieved in the dark-growing angelica cell suspension cultures cultured in the hormone free medium. Removal of growth regulators from the culture medium brought about 5-fold increase in coumarins, and by combining hormonal deprivation and fungal elicitation, a 15-fold enhancement of coumarins was achieved, in comparison with control cultures cultured in the presence of plant growth regulators and without elicitor treatment.
Key words: *Angelica archangelica* L. – cell suspension cultures – growth – coumarins – growth regulators – light – elicitation – *Pythium* – flow injection analysis

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SOUHRN

Vliv kombinovaného odnětí hormonů a houbového elicitoru na produkci kumarinů v suspenzní kultuře *Angelica archangelica* L.
Účinnost elicitace jako nástroje pro zvýšení produkce sekundárních metabolitů v rostlinných explantátových kulturách závisí na komplexní interakci mezi elicitorem a rostlinnou buňkou. V této práci byl zkoumán vliv odstranění rostlinných růstových regulátorů a světelných podmínek na buněčný růst a elicitaci kumarinů v suspenzní kultuře *Angelica archangelica*. Jako elicitor byl použit autoklávovaný homogenát houby *Pythium aphanidermatum*. Buněčné kultury *Angelica archangelica* byla kultivovány v médiu s přídavkem kyseliny 2,4-dichlorfenoxyoctové a benzylaminopurinu nebo bez hormonů, ve tmě nebo za stálého osvětlení. Růst kultur (čerstvá i suchá hmota) nebyl ve srovnání s kontrolní kulturou přítomností houbového elicitoru nepříznivě ovlivněn. Obsah kumarinů v buňkách byl ovlivněn málo. Obsah kumarinů v médiu byl stimulován odnětím hormonů a elicitací v závislosti na světelných podmínkách; nejlepší výsledky

Address for correspondence:
PharmDr. Tomáš Siatka, CSc.
Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Department of Pharmacognosy, Czech Republic
Heyrovského 1203, 500 05 Hradec Králové
e-mail: siatka@faf.cuni.cz

byly dosaženy v kulturách andělíky rostoucích ve tmě v médiu bez hormonů. Odstranění růstových regulátorů z kultivačního média vyvolalo pětinásobné zvýšení kumarinů, kombinací hormonální deprivace a elicitace bylo dosaženo patnáctinásobného zvýšení kumarinů ve srovnání s kontrolní kulturou kultivovanou v přítomnosti rostlinných růstových regulátorů a bez přídavku elicitoru.

Klíčová slova: *Angelica archangelica* L. – suspenzní kultura – růst – kumariny – růstové regulátory – světlo – elicitace – *Pythium* – průtoková injekční analýza

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Introduction

Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavours, and other industrial materials. Plant cell culture technology shows promise for a large-scale production of valuable plant products. However, it still faces many biological and biotechnological limitations. One of the major obstacles is a low yield of plant secondary metabolites in plant cell cultures [1, 3]. Many approaches have been developed to overcome this problem [2, 3], the most notable strategy for improving metabolite yields perhaps being elicitation [4]. Elicitors are chemicals or biofactors from various sources that can induce an upregulation of genes. Some elicitors target secondary metabolic genes, which are often associated with defence responses to perceived environmental changes [1, 4] Elicitors may be biotic or abiotic. The biotic elicitors are of biological origin, derived from a pathogen (fungi, bacteria, viruses or herbivores) or from the plant itself (e.g., plant cell wall components). Biotic compounds can be of defined composition, when their molecular structures are known, or have a complex composition when they comprise several different molecular classes making it impossible to define a unique chemical identity (e.g., fungal homogenates or culture filtrates). On the other hand, abiotic elicitors are not of a biological origin and are grouped in physical factors (such as thermal and osmotic stress, radiation, and wounding) and chemical compounds (e.g., heavy metal salts) [5]. It is well known that treatment with elicitors causes an array of defence reactions, including the accumulation of secondary metabolites in intact plants [6–8] as well as in cell cultures [9–11]. The effectiveness of elicitation as a tool to enhance the production of secondary metabolites depends on a complex interaction between the elicitor and the plant cell. The main factors that can affect this interaction and thereby the elicitation response include elicitor specificity, elicitor concentration and elicitation time, and culture conditions (such as the growth stage, medium composition, and light) [5].

We report here the effects of elicitor concentration, removal of plant growth regulators, and light conditions on the cell growth and elicitation of coumarins in *Angelica archangelica* cell suspension cultures. An autoclaved homogenate of *Pythium aphanidermatum* was used as the elicitor.

EXPERIMENTAL PART

Chemicals

2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine (Sigma, St. Louis, U.S.A.); scopoletin (Fluka, Buchs, Germany); dibasic sodium phosphate and monobasic potassium phosphate (Lachema, Brno, Czech Republic).

Instruments

A PS 20A autoclave (Chirana, Brno, Czech Republic); a roller (Vyvojove dilny, Academy of Sciences of the Czech Republic, Praha, CR); a LGA 05 lyophilizer (Janetzki, Leipzig, Germany); a 200S analytical scale (Sartorius, Göttingen, Germany); a single-channel plunger LPC 3001 micropump, a PP05 peristaltic pump for sample delivery, and a TZ 4620 chart recorder (Laboratorni pristroje, Praha, CR); a home-made PTFE valve with exchangeable sample loops; and a Schoeffel FS 970 fluorescence detector (McPherson, Chelmsford, U.S.A.).

Cell suspension cultures and elicitor treatment

Cell suspension cultures of *Angelica archangelica* were established as described previously[12] and grown in liquid Murashige and Skoog medium [13] supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.4 mg/l benzylaminopurine (BA), and 30 g/l sucrose. They were agitated in 250 ml flasks containing 30 ml of the medium on a roller apparatus at 8 rpm, incubated at 25 °C under continuous light (3,500 lux) or in darkness, and subcultured every 14 days. The culture used in this study was kept *in vitro* for three years.

The freeze-dried powdered mycelia of *Pythium aphanidermatum* were suspended in distilled water, autoclaved (121 °C, 15 min), and appropriate amounts (0.5, 1, 5, 10, and 50 mg) in 2 ml per flask were added to angelica cell cultures on day 10 following the transfer to a fresh medium. Control cultures received 2 ml of sterile distilled water. For testing the effects of hormonal deprivation on the elicitation, cell cultures were grown as one subculture (14 days) in a medium without 2,4-D and BA, and

Table 1. Effect of Pythium aphanidermatum elicitor on biomass accumulation and production of coumarins in Angelica archangelica cell suspension cultures grown under dark and light conditions in the medium supplemented with growth regulators (2,4-D and BA)
*(Data represent as the means with standard deviations. Values with asterisks * are significantly higher in comparison with control cultures, P < 0.05)*

Elicitor amount (mg/flask)	Cultures in the dark				Cultures in the light			
	Culture growth		Content of coumarins		Culture growth		Content of coumarins	
	Fresh weight (g/flask)	Dry weight (mg/flask)	Cells (mg/g dry weight)	Medium (mg/l)	Fresh weight (g/flask)	Dry weight (mg/flask)	Cells (mg/g dry weight)	Medium (mg/l)
0	4.40 ± 0.88	220 ± 42	0.48 ± 0,06	0.81 ± 0.08	2.80 ± 0.41	241 ± 27	0.23 ± 0.03	1.43 ± 0.15
0.5	5.20 ± 0.82	286 ± 60	0.38 ± 0.05	0.82 ± 0.06	2.75 ± 0.20	234 ± 22	0.23 ± 0.02	1.88 ± 0.18*
1	4.92 ± 0.74	236 ± 44	0.36 ± 0.05	0.81 ± 0.09	2.70 ± 0.24	236 ± 33	0.27 ± 0.03	1.79 ± 0.16*
5	5.60 ± 0.98	272 ± 46	0.30 ± 0.06	0.86 ± 0.07	2.90 ± 0.45	244 ± 41	0.28 ± 0.04	1.94 ± 0.27*
10	4.20 ± 0.72	238 ± 56	0.29 ± 0.04	0.92 ± 0.04	2.70 ± 0.16	222 ± 26	0.29 ± 0,03	2.04 ± 0.24*
50	4.60 ± 0.66	216 ± 22	0.45 ± 0.08	1.27 ± 0.08*	3.30 ± 0.57	220 ± 33	0.34 ± 0.05*	2.68 ± 0.29*

Table 2. Effect of Pythium aphanidermatum elicitor on biomass accumulation and production of coumarins in Angelica archangelica cell suspension cultures grown under dark and light conditions in the hormone free medium
*(Data represent as the means with standard deviations. Values with asterisks * are significantly higher in comparison with control cultures, P < 0.05)*

Elicitor amount (mg/flask)	Cultures in the dark				Cultures in the light			
	Culture growth		Content of coumarins		Culture growth		Content of coumarins	
	Fresh weight (g/flask)	Dry weight (mg/flask)	Cells (mg/g dry weight)	Medium (mg/l)	Fresh weight (g/flask)	Dry weight (mg/flask)	Cells (mg/g dry weight)	Medium (mg/l)
0	5.00 ± 0.57	361 ± 28	0.47 ± 0.05	4.14 ± 0.43	3.90 ± 0.57	385 ± 65	0.38 ± 0.05	2.84 ± 0.29
0.5	5.25 ± 0.45	360 ± 47	0.60 ± 0.07	6.38 ± 0.38*	3.50 ± 0.41	327 ± 43	0.53 ± 0.07*	5.21 ± 0.57*
1	5.45 ± 0.53	356 ± 22	0.58 ± 0.06	7.61 ± 0.54*	3.60 ± 0.37	335 ± 42	0.41 ± 0.06	4.95 ± 0.45*
5	5.30 ± 0.33	357 ± 38	0.54 ± 0.05	8.34 ± 0.81*	3.75 ± 0.29	291 ± 51	0.48 ± 0.05	5.52 ± 0.61*
10	5.15 ± 0.49	350 ± 50	0.56 ± 0.04	9.52 ± 0.86*	3.30 ± 0.49	295 ± 49	0.37 ± 0,04	4.71 ± 0.49*
50	5.20 ± 0.20	342 ± 30	0.58 ± 0.07	12.36 ± 1.22*	3.40 ± 0.53	287 ± 47	0.40 ± 0.06	3.45 ± 0.37

then, in the second subculture in the medium free of growth regulators, were elicited on day 10 as mentioned above.

Elicited and control cultures were harvested 48 hours after treatment, and the growth of cultures and production of coumarins were evaluated.

Analytical procedures

Cells were separated from the culture medium by vacuum filtration using a Buchner funnel with filter paper.

Filtered cells were weighed for fresh weight determination, and then freeze-dried to obtain dry weight.

Coumarins in freeze-dried cells and in the culture medium were quantified fluorometrically by flow injection analysis as described in detail previously [14].

All data presented are the mean values of three replicates with standard deviations. Student's t-test was used for statistical analysis of data, and differences with $P < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

In view of the variability in elicitation responses due to different factors, the optimization of medium composition and culture conditions represent an important aspect in elicitation protocols [5]. Growth regulators [15–17] as well as light [18–20] influence the metabolism of plant cells, and therefore, we examined the effects of hormonal deprivation and light conditions on the elicitation of coumarins in *Angelica archangelica* suspension cultures after addition *Pythium aphanidermatum* homogenate as the elicitor. The cultures were cultured in a medium supplemented with 2,4-dichlorophenoxyacetic acid and benzylaminopurine or in a hormone free medium, and in the dark or under continuous light.

The culture growth (both fresh and dry biomass) was not affected adversely in the presence of the fungal elicitor as compared with the control cultures (Tables 1 and 2), independently of the medium composition or light conditions. Similarly to *Angelica archangelica*

suspension cultures, the fungal elicitor did not influence the growth in *Sanguinaria canadensis* cell cultures grown in a medium with or without 2,4-dichlorophenoxyacetic acid [17].

As for the production of coumarins in *Angelica archangelica* suspension cultures, a better response to the fungal elicitor treatment was achieved in the hormone free medium. In cultures grown in the medium with growth regulators (Table 1), enhanced accumulation of coumarins was found in the culture medium in the light. This was a 1.8-fold increase over the untreated control at the highest elicitor concentration. In cultures grown in the medium without growth regulators (Table 2), the contents of coumarins in cells were influenced only slightly under both light and dark conditions. The highest yields of coumarins were achieved in the culture medium in the dark-growing cultures; the level of coumarins rose with an increasing concentration of the fungal elicitor up to a threefold amount compared with the control cultures. Similar findings regarding the effects of combined hormonal deprivation and fungal elicitation have been reported on alkaloid accumulation in *Sanguinaria canadensis* cell suspension cultures [17]. Our results are in agreement with those observed in *Hypericum perforatum* cell suspension cultures where the production of hypericin was stimulated with different effectiveness after elicitor treatment depending on light conditions [18].

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REFERENCES

1. **Zhao, J, Davis, L. C., Verpoorte, R.:** Biotechnol. Adv., 2005; 23, 283.
2. **Roberts, S. C.:** Nat. Chem. Biol., 2007; 3, 387.
3. **Roberts, S. C., Shuler, M. L.:** Curr. Opin. Biotechnol., 1997; 8, 154.
4. **Kolewe, M. E., Gaurav, V., Roberts, S. C.:** Mol. Pharm., 2008; 5, 243.
5. **Vasconsuelo, A., Boland, R.:** Plant Sci., 2007; 172, 861.
6. **Bednarek, P., Osbourn, A.:** Science, 2009; 324, 746.
7. **Ziaratnia, S. M., Kunert, K. J., Lall, N.:** S. Afr. J. Bot., 2009; 75, 97.
8. **Mandal, S., Mitra, A.:** Physiol. Mol. Plant Pathol., 2007; 71, 201.
9. **Ferri, M. et al.:** Proteomics, 2009; 9, 610.
10. **De Alwis, R. et al.:** J. Plant Physiol., 2009; 166, 720.
11. **Roat, C. Ramawat, K. G.:** Plant Biotechnol. Rep., 2009; 3, 135.
12. **Siatka, T., Kašparová, M.:** Čes. slov. Farm., 2008; 57, 17.
13. **Murashige, T., Skoog, F.:** Physiol. Plant., 1962; 15, 473.
14. **Siatka, T., Solich, P., Kotyk, R.:** Pharmazie, 1998; 53, 273.
15. **Santner, A., Calderon-Villalobos, L. I. A., Estelle, M.:** Nat. Chem. Biol., 2009; 5, 301.
16. **Jeong, G.-T, Woo, J-C., Park, D.-H.:** Biotechnol. Bioprocess Eng., 2007; 12, 86.
17. **Cline, S. D., McHale, R. J., Coscia, C. J.:** J. Nat. Prod., 1993; 56, 1219.
18. **Walker, T. S., Bais, H. P., Vivanco, J. M.:** Phytochemistry, 2002; 60, 289.
19. **Jacques, P. et al.:** Acta Bot. Gall., 2007; 154, 21.
20. **Ceoldo, S. et al.:** Plant Sci., 2009; 176, 553.