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Stimulation of furanochromone accumulation in callus cultures of *Ammi visnaga* L. by addition of elicitors

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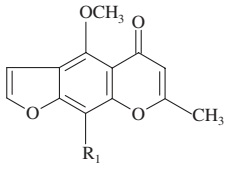
In order to check the possibility of producing secondary metabolites, *in vitro* cultures of *A. visnaga* callus were established. The best growth of *A. visnaga* callus was obtained on Murashige and Skoog medium (MS) containing 6-benzyladenine (BA) and α-naphtaleneacetic acid (NAA). The study was concentrated on the induction of production of secondary metabolites by exposing callus to abiotic elicitors: benzo(1,2,3)-thiadiazole-7-carbothionic acid *S*-methyl ester (BION®) and a suspension of silica (SiO_2) and biotic elicitors: autoclaved lysates of *Enterobacter sakazaki* and scleroglucan. GC analysis indicated that not-elicited callus of *A. visnaga* grown in darkness accumulated 2 times more visnagin than the one which was grown under a 16-h photoperiod. The highest accumulation of visnagin was observed in the callus culture elicited with scleroglucan or BION®. Scleroglucan induced also the accumulation of khellin in *A. visnaga* callus. The presented work shows that biosynthesis of pharmacologically important secondary metabolites in *A. visnaga* cultures could be stimulated by application of elicitors.

1. Introduction

Plant tissues produce a large number of metabolites which are used in pharmaceutical industries. Toothpick Ammi (*Ammi visnaga* L.) family *Apiaceae*, is a subtropical, mediterranean annual plant of about 1–1.5 m hight. It grows in the Middle East and is collected particularly in Egypt, Canary Ills. and Iran. The attempts to acclimatise this plant in Europe were not successful because of the cool climate (light frosts) which caused limited seed production and poor germination rate [1, 2]. The seeds of *A. visnaga* contain furanochromones such as khellin (0.5–1.5%) and visnagin (0.05–0.1%), and secondary pyranocoumarins, such as visnadin and samidyn [3]. The extract of dried seeds of *A. visnaga* has been used as an antispasmodic drug in the treatment of angina pectoris [4], cardiovascular diseases and urolithiasis [5]. Studies on the photogenic and mutagenic activity of visnagin have also been reported [6]. The plant derived preparation which based on furanocoumarins from *A. visnaga* is Tinctura Ammi visnagae.

The furanochromones – visnagin and khellin – have a very limited distribution in the plant kingdom and are reported only in *A. visnaga* and *Eranthis hyemalis* L. (Ranunculaceae) [7, 8]. The pharmacological activities of these furanochromones and the restricted area in which this plant grows has raised the interest in developing a biotechnological approach for their production. The study of Kaul and Staba [9] indicated that *in vitro* cultures of *A. visnaga* growing on MS medium are not an efficient source for production of visnagin and khellin. According to Verpoorte et al. [10] a strategy which can improve the productivity of *in vitro* grown cultures is the induction or stimulation of the secondary metabolite accumulation by the application of elicitors. Elicitors are compounds which stimulate accumulation of the new compounds in the stressed or infected tissue [10, 11]. Study of *in vitro* cultures of different species from the family *Apiaceae* showed indeed that accumulation of secondary metabolites can be elicited by application of abiotic and biotic elicitors, e.g. celery [12], cucumber [13], parsley [14], carrot and parsley [2] and Bishops weed [15]. The aim of this research was to develop an efficient method for induction of furanochromone production in *A. visnaga* callus cultures.



	R_1
Khellin	OCH_3
Visnagin	H

2. Investigations, results and discussion

A. visnaga callus cultures were derived from the hypocotyl of sterile seedlings. Explants were placed on MS medium supplemented with different level of growth regulators: 0.1–5.0 mg/l NAA; 0.1–5.0 mg/l IAA; 0.1–5.0 mg/l 2,4-D; 0.1–2.0 mg/l BA; 0.1–4.0 mg/l KIN; 0.5–2.0 mg/l zeatin. The best growth of callus was obtained on

Table 1: Influence of light and elicitors on growth of *A. visnaga* callus

	Biomass callus cultures after 18 days growth (dry weight, mg)
Callus in the dark	90 ± 2
Callus under a 16-h photoperiod	122 ± 4
Callus[a] + SiO_2	35 ± 2
Callus[a] + BION®	160 ± 8
Callus[a] + *E. sakazaki*	45 ± 3
Callus[a] + scleroglucan	183 ± 6

Callus cultures were grown on for 18 days on MS medium supplemented with 2.5 mg/l NAA, 1.0 mg/l BA, 3% sucrose and 0.75% agar. Initial weight of explant placed on MS medium was about 28 mg
[a] Callus growing under a 16-h photoperiod

MS medium [16] containing 2.5 mg/l NAA and 1.0 mg/l BA.

Growth of callus cultures was estimated under a 16-h photoperiod and in the dark. An initial weight of explant was 28 mg. A higher growth rate was obtained for callus growing under a 16-h photoperiod (Table 1). Kaul and Staba [7, 9] recommended the same medium but supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). However, Supniewska and Dohnal [17] proposed Woody medium supplemented with NAA and kinetin for efficient growth of *A. visnaga* callus.

Earlier experiments did not indicate significant differences in the content of secondary metabolites in *A. visnaga* callus cultures growing on MS medium supplemented with different levels of growth regulators (0.1–5.0 mg/l NAA; 0.1–5.0 mg/l IAA; 0.1–5.0 mg/l 2,4-D; 0.1–2.0 mg/l BAP; 0.1–4.0 mg/l KIN; 0.5–2.0 mg/l zeatin) and trophic compounds (coconut water, meso-inositol) [18]. Analysis indicated that callus of *A. visnaga* grown in the dark accumulated about two times more visnagin (65 μg/g d.w.) than that grown under a 16-h photoperiod (32 μg/g d.w.) (Table 2). Khellin was not detected in samples growing in dark. However a low level of this compound was detected under a 16-h photoperiod (Table 2). Other authors indicated that *in vitro* cultures of *A. visnaga* (callus and hairy roots) synthesised visnagin but not khellin [7, 19]. In order to improve the production of the compound of interest two abiotic and two biotic elicitors were applied.

The growth rates of elicited and not elicited tissues were significantly different (Table 1). An increase in fresh weight of elicited callus was observed after application of BION® and scleroglucan (1.3 and 1.5 times, respectively). However, a significant decline of growth was noticed when SiO_2 and autoclaved lysates of *E. sakazaki* (3.5 and 2.7 times, respectively) were added to MS medium (Table 1).

Table 2: Comparison of furanochromones level in control and elicited callus of *A. visnaga*

Kind of culture	Visnagin	Khellin
	(μg/g of dry weight)	
Callus, 16-h photoperiod	32 ± 5	7 ± 2
Callus in the dark	65 ± 11	n.d.
Callus, 16-h photoperiod + BION®	112 ± 21	n.d.
Callus, 16-h photoperiod + SiO_2	29 ± 4	n.d.
Callus, 16-h photoperiod + *E. sakazaki*	14 ± 2	n.d.
Callus, 16-h photoperiod + scleroglucan	157 ± 25	17 ± 2

Callus cultures were grown on for 18 days on MS medium supplemented with 2.5 mg/l NAA, 1.0 mg/l BA, 3 % sucrose and 0.75% agar.
Callus growing under a 16-h photoperiod.
n.d. not detected

The highest level of visnagin was determined in the extract of callus grown under a 16-h photoperiod on MS media with scleroglucan (5 times more than control callus), Table 2. This callus also showed the highest growth rate. Treatment with scleroglucan slightly stimulated accumulation of khellin (Table 2).

Induction of visnagin accumulation was observed also in callus treated by BION® (3.5 times more than control callus) (Table 2). Application of autoclaved lysates of *E. sakazaki* and silica to callus cultures of *A. visnaga* did not induce visnagin production (Table 2).

Earlier data showed that the treatment of callus cultures of *A. visnaga* with acetylsalicylic acid or jasmonic acid leads to a slight accumulation of the furanochromone visnagin and the pyranocoumarin samidin [20].

The presented study shows that elicitor treated *in vitro* cultures of *A. visnaga* could be an alternative source of furanochromones. The biosynthesis of visnagin in *A. visnaga* cultures could be induced by scleroglucan or BION®. Applied elicitors not only increased the production of visnagin but also induced the growth rate of callus cultures. Furthermore, elicitation of visnagin production in *in vitro* cultures of *A. visnaga* can serve as a useful tool for analysing the biosynthesis of furanochromones in plant tissues.

3. Experimental

3.1. In vitro culture of A. visnaga

Different methods for seed sterilisation and propagation of *in vitro* plants directly from seeds were checked, but all of them were ineffective (70% alcohol, 5–10% chloral, chloramin T, clotrimazol, actidion). We were not able to germinate seeds on media with sucrose because of contamination with fungi. Apparently seeds were infected inside the seedcoat. Finally *A. visnaga in vitro* cultures were initiated from plants growing in a greenhouse. An effective method for sterilisation of explants was worked out [18].

The plantlets of *A. visnaga* were grown in MS medium [16] supplemented with 2.0 mg/ml NAA, 1.0 mg/l BA, 3% sucrose and solidified with 0.75% agar, at a temperature of 20–22 °C and illumination of 1200 lux with a photoperiod of 16 h light/8 h dark for 3–4 weeks [18]. The pH of the media was adjusted to 5.8 prior to autoclaving. Callus induction and growth was obtained also in MS medium but supplemented with NAA (2.5 mg/l) and BA (1.0 mg/l) [18].

3.2. Induction of secondary metabolites using abiotic and biotic elicitors

Two types of abiotic elicitors (silicon dioxide SiO_2 and BION®) and biotic elicitors (*Enterobacter sakazaki* and scleroglucan – polysaccharide obtained from cell wall of *Sclerotina gluconicum*) were used.

Well grown 4-week old callus was passaged on MS medium supplemented with 2.5% SiO_2 (Sigma). BION®, a synthetic activator of plant systemic acquired resistance (SAR) [14], obtained from Novartis Poland Warsaw, was added to MS medium (final concentration 2.5 mg/l MS). Both elicitors were added to MS medium before autoclaving.

Biotic elicitor, 0.01% solution of scleroglucan, was added to MS medium on which callus cultures were placed and grown for 4 weeks. Cultures of *Enterobacter sakazaki* (family *Enterobacteriaceae*) [20], were grown in LB medium [21] in darkness at 37 °C for 24 h. The suspension of bacteria was treated with toluene (100 : 1) and autoclaved (30 min/1 atm). Before autoclaving samples were left for 1 h for toluene evaporation. Suspension of *E. sakazaki* cell extract, $OD_{600} = 1.2$ (15 ml/l) was added to MS medium on which callus cultures were placed and grown for 4 weeks.

3.3. Determination of furanochromones

Extraction of the secondary metabolites was performed 4 weeks after the treatment with elicitors. Samples of not-elicited and elicited callus were dried and extracted exhaustively with petrol ether, chloroform and methanol using a Soxhlet apparatus. Chloroform extracts were evaporated and purified on a silica gel column [22]. The samples were afterwards analysed by GC and GC-MS. GC analyses were carried out on a GC 8000 TOP gas chromatograph, equipped with a capillary column (DB1-HT, length 30 m, I. D. 0.25 mm, 0.1 μm film thickness) with split ratio 1 : 30 for an injec-

tion port. Argon was used as a carrier gas [23]. Initial oven temperature was 100 °C kept for 10 min, then a temperature programme of 4 °C/min was employed up to 320 °C. Methoxynaphtalene was used as an internal standard in this stage of experiments.
MS (70 eV) were recorded on a VG TRIO-2000 mass spectrometer. The samples were introduced via Hewlett Packard 5890 gas chromatograph equipped with RTX-1 column and under the same chromatographic conditions as mentioned for GC. Helium was used as a carrier gas.

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References

1 Ożarowski, A.: Państwowy Zakład Wydawnictw Lekarskich, Warszawa 1983
2 Ekiert, H.: Pharmazie **55**, 561 (2000)
3 Evans, W. C.: WB Saunders Company Ltd, 421 (1989)
4 Duarte, J.; Vallejo, I.; PerezVizcaino, F.; Jimenez, R.; Zarzuelo, A.; Tamargo J.: Planta Med. **63**, 233 (1997)
5 Rauwald, H.; Brehm, O.; Odenthal, K. P.: Planta Med. **60**, 101 (1994)
6 Schimmer, O.: Mutagenesis **12**,141 (1997)
7 Kaul, B.; Staba, E. J.: Planta Med. **15**, 145 (1967)
8 Kohlmünzer, S.: Wydawnictwo Lekarskie PZWL, Warszawa 1998
9 Kaul, B.; Staba, E. J.: Science **150**, 1731 (1965)
10 Verpoorte, R.; Van der Heijden, R.; Hoopen, H. J. G.; Memelink, J.: Biotechnol. Lett. **21**, 467 (1999)
11 Pitta-Alvarez, S. I.; Spollansky, T. C.; Giulietti, A. M.: Enzyme Microb. Technol. **26**, 252 (2000)
12 Miksch, M.; Boland, W.: Experientia **52**, 739 (1996)
13 Fawe, A.; Abou-Zaid, M.; Menzies, J. G.; Belanger, R. R.: Phytopathology **88**, 396 (1998)
14 Katz, V. A.; Thulke, O. U.; Conrath, U.: Plant Physiol. **117**, 1333 (1998)
15 Królicka, A.; Staniszewska, I.; Krauze-Baranowska, M.; Maliński, E.; Szafranek, J.; Turkiewicz, M.; Łojkowska, E.: Acta Horticulturae **560**, 255 (2001)
16 Murashige, T.; Skoog, F.: Physiol. Plantarum **15**, 473 (1962)
17 Supniewska, J.; Dohnal, B.: Acta Soc. Bot. Polon. **66**, 559 (1977)
18 Królicka, A.: Doctor's Thesis, University of Gdańsk, Poland 1999
19 Kursinszki, L.; Troilina, J.; Szõke, É.: Microchem. J. **59**, 392 (1998)
20 Krieg, N. R.; Holt, J. G.; Willkins, W. (eds.): Bergey's Manual of Systematic Bacteriology
21 Maniatis, T.; Fritsch, E. F.; Sambrook, J.: Cold Spring Harbour Laboratory, Cold Spring N.Y. 1982
22 Spencer, G. F.; Tjarks, L. W.; Powell, R. G.: J. Agric. Food Chem. **35**, 803 (1987)
23 Królicka, A.; Staniszewska, I.; Bielawski, K.; Maliński, E.; Szafranek, J.; Łojkowska, E.: Plant Sci. **160**, 259 (2001)