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Furanocoumarins in *Pastinaca sativa* L. *in vitro* culture

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Callus cultures of *Pastinaca sativa* L. (parsnip), Apiaceae, were cultivated on variants of Linsmaier-Skoog's medium, containing varying quantities (0.1–10.0 mg/l) of phytohormones: NAA-BAP and IBA-BAP which allowed to obtain 1.5–3-fold fresh biomass growth during 6-week subcultures. HPLC analyses showed that tissues cultured *in vitro* produced psoralen, bergapten, xanthotoxin, isopimpinellin and umbelliferone which are well known metabolites in plants growing under natural conditions. Total content of coumarins depended on the nature and quantity of phytohormones present in the medium, and ranged from 115.7 to 408.5 mg/100 g of the dry weight, isopimpinellin being the metabolite which dominated quantitatively (maximum content of 238.9 mg/100 g). Psoralen was also accumulated in callus tissues at considerable amounts (maximum content of 108.8 mg/100 g). This metabolite dominated in vegetative plant parts that have been analysed in our study (leaves, stems, roots) but its contents were lower than in the material from *in vitro* culture (48.9 mg/100 g 10.6 mg/100 g and 14.9 mg/100 g, respectively). Imperatorin was not detected in callus tissues although it dominated in the analysed fruits of the studied plant (200.0 mg/100 g). The best of the tested media in respect of promoting tissue biosynthetic capabilities was that which contained 3 mg/l NAA and 1 mg/l BAP. The studies showed that *in vitro* cultures of *Pastinaca sativa* L. can be a convenient model to study the biosynthesis of furanocoumarins and also a potential rich source of these compounds, particularly isopimpinellin.

1. Introduction

Pastinaca sativa L., parsnip (Apiaceae), is a valuable species because of its therapeutic potential and as legume as it is rich in coumarin compounds, flavonoids, vitamins, sugars, proteins, lipids and mineral components [1, 2]. The main representatives of coumarin compounds are furanocoumarins with linear structure: psoralen, bergapten, xanthotoxin, isopimpinellin, imperatorin and the angular furanocoumarin sphondin [3–6].

Furanocoumarins, especially those with linear structure, are interesting compounds because of their photosensitising and antiproliferative actions [7]. They were the subject of the present work.

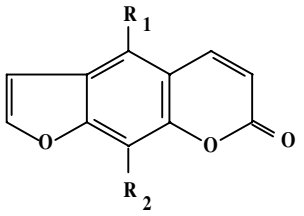
Pastinaca sativa callus cultures were hitherto studied in terms of localization of furanocoumarins in tissues [8], and growth capabilities of tissues of various origins (cultures derived from leaves, roots, stems) under different conditions of *in vitro* culture [9, 10]. Detailed studies on the accumulation of coumarin compounds have not been conducted so far. This paper presents results of HPLC analyses of tissue material collected from callus cultures cultivated on various variants of Linsmaier-Skoogs medium, and vegetative parts of the plant (leaves, stems, roots) and fruits chosen as reference material.

2. Investigations, results and discussion

Hypocotyl parts of sterile seedlings proved to be the best explants for establishment of callus cultures.

Linsmaier-Skoog's medium (L-S) [11] containing 2 mg/l NAA and 2 mg/l BAP proved beneficial both for callus initiation and maintaining tissue growth. We obtained similar results in the studies of callus cultures of *Ammi majus* L. [12] and *Anethum graveolens* L. (unpublished) and shoot cultures of *Ruta graveolens* L. [13].

The tested variants of L-S medium allowed to obtain an 1.5–3-fold increase in callus tissue fresh biomass of *Pastinaca sativa* during a 6-week subculture. The increments were low in comparison with the increase in callus tissue biomass of *Ammi majus* on identical media (in which a 2.3–6.6-fold biomass gain was observed on the average) [12]. Some *Pastinaca sativa* callus tissues showed the tendency to develop overground shoots during successive subcultures. As a result, two different cultures were obtained, callus and shoot cultures, which differed in the degree of cell differentiation. The shoot culture was maintained only on one of the tested media (2 mg/l NAA, 2 mg/l BAP) yielding similar biomass increments as in the case of callus tissue.



	R_1	R_2
Psoralen	H	H
Xanthotoxin	H	OCH_3
Bergapten	OCH_3	H
Isopimpinellin	OCH_3	OCH_3
Imperatorin	H	$OCH_2-CH=C(CH_3)_2$
Umbelliferone (7-hydroxycoumarin)		

HPLC analysis of the extracts of tissue material from *in vitro* cultures showed the presence of five coumarin compounds: psoralen, bergapten, xanthotoxin, isopimpinellin and umbelliferone. The mentioned furanocoumarins are common metabolites in the parent plant [3–6]. The presence of all these furanocoumarins was also demonstrated in leaf and fruit extracts which were used as reference material in our studies. Imperatorin was not detected in callus tissue although it is usually abundant in plants growing under natural conditions [5, 6]. The presence of this metabolite was also detected in our plant extracts. Contents and spectrum of coumarin compounds in the material derived from *in vitro* cultures were different than those found in material grown under natural conditions (Fig., Table 1). The metabolite which dominated quantitatively in the material from *in vitro* culture was isopimpinellin (independently of the medium composition)

Table 1: Contents of the metabolites in different vegetative parts and fruits of *Pastinaca sativa* L. and their maximum amounts obtained in *in vitro* cultures on various media.

Metabolites	Content (mg/100 g of dry weight)					
	In vitro cultures		Fruits	Leaves	Stems	Roots
	Callus tissues*	Shoots**				
Psoralen	108.82	5.77	28.90	48.90	10.60	14.90
Bergapten	16.15	0.58	24.06	8.60	–***	–
Xanthotoxin	82.62	6.90	1.77	3.20	–	5.60
Isopimpinellin	238.88	24.02	8.02	4.80	–	6.40
Imperatorin	–	1.67	200.00	7.80	1.50	0.06
Umbelliferone	1.80	0.33	–	–	–	–

* callus tissues cultivated on various variants of L-S medium
** shoots cultivated *in vitro* on one variant of L-S medium (2 mg/l NAA, 2 mg/l BAP)
*** content lower than 0.001 mg/100 g

with contents ranging from 90.2 to 238.9 mg/100 g of the dry weight (contents of furanocoumarins are always expressed in this way further on in this paper). Contents of psoralen and xanthotoxin were also high amounting to 18.6–108.8 mg/100 g and 14.1–82.6 mg/100 g, respectively. Bergapten amounts were low (7.4–16.1 mg/100 g). Umbelliferone, studied as biogenetic precursor of furanocoumarins, was detected only in the material from four media at very low quantities (0.5–1.8 mg/100 g). The results of HPLC analysis of callus tissue derived from consecutive subcultures (10^{th}, 12^{nd}–14^{th}) on the same variant of L-S medium (2 mg/l NAA, 2 mg/l BAP) showed similar levels of accumulation of the metabolites (Table 2).

The contents of the studied coumarins in shoots cultivated *in vitro* were lower than in callus tissue (maximum total content of 71.5 mg/100 g) in spite of their high degree of differentiation, and this was the reason why this material has not been studied further during the present study (Table 1).

Imperatorin was the dominating metabolite in the studied fruits (200.0 mg/100 g). Bergapten and psoralen were accumulated at approximately 7–8-fold lower quantities in comparison with imperatorin. Xanthotoxin and isopimpinellin contents in fruits were very low. Studies by other authors [5, 6] indicated that imperatorin or xanthotoxin were the metabolites which were accumulated at the highest amounts in fruits that is in line with our findings. Contents of isopimpinellin in fruits were usually lower. Fruits are plant organs characterised by the highest accumulation of furanocoumarins. Contents of these metabolites in vegetative plant parts were found to be lower [4]. Our studies confirmed these results. Moreover, the other metabolite, psoralen prevailed as well in leaf and stem as root extracts (48.8 mg/100 g, 10.6 mg/100 g and 14.9 mg/100 g, respec-

Table 2: Contents of the metabolites in callus tissues from consecutive subcultures on L-S medium (2 mg/l NAA, 2 mg/l BAP)

Metabolites	Content (mg/100 g of dry weight)			
	Subculture 10	Subculture 12	Subculture 13	Subculture 14
Psoralen	18.61	38.41	51.13	22.36
Bergapten	5.01	12.34	10.07	8.60
Xanthotoxin	44.38	46.52	45.48	38.80
Isopimpinellin	158.99	231.95	176.83	159.18
Imperatorin	–*	–	–	–
Umbelliferone	–	–	–	–

* content lower than 0.001 mg/100 g

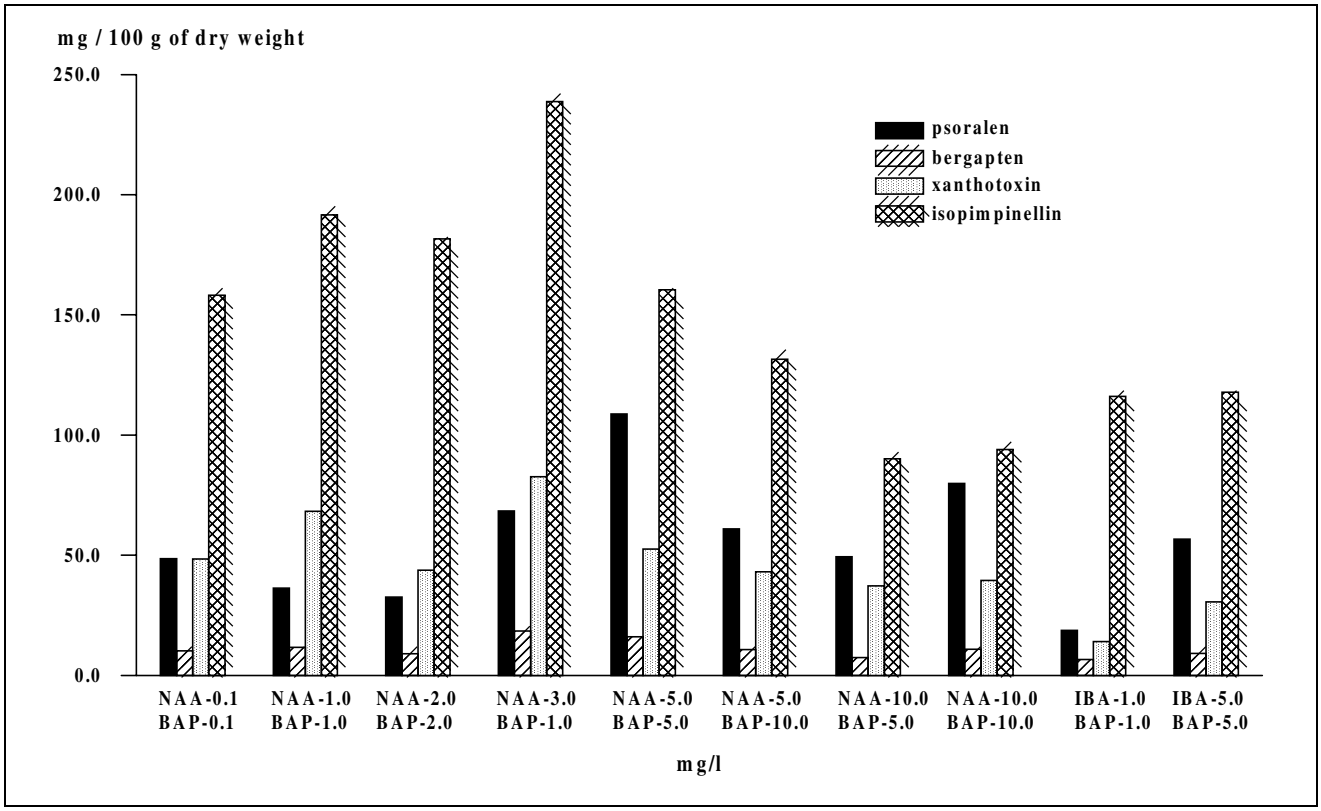


Fig.: Contents (mg/100 g of dry weight) of the studied coumarin compounds in callus tissues of *Pastinaca sativa* L. cultivated on media with different concentrations of phytohormones [mg/l]. Due to the low contents of umbelliferone (0–1.8 mg/100 g) detected only on four of the tested media, this metabolite was not included into the figure.

tively). Accumulation of the remaining furanocoumarins was lower than 10 mg/100 g.

Different qualitative and quantitative composition of secondary metabolites in *in vitro* cultures in comparison with plant material grown under natural conditions is a common phenomenon. For example, our research on *Ammi majus* showed that marmesin and xanthotoxin prevailed in plants growing in open air while isopimpinellin, imperatorin and bergapten dominated in callus tissue [12].

The total content of the studied metabolites in *Pastinaca sativa* approximating 0.4 g/100 g is an interesting value. There are only a few examples of such biosynthetic efficacy in undifferentiated callus tissues. These values are much higher that those which we obtained in *Ammi majus* (152.8 mg/100 g) [12]. Most studies on furanocoumarin biosynthesis in *in vitro* cultures in species of the *Apiaceae* family have suggested the application of biological elicitors (*Phytophthora megasperma* f. sp. *glycinea*, *Alternaria carthami* and scleroglucan from *Sclerotium sclerotiosum*) to stimulate biosynthesis of these compounds, as furanocoumarins are considered to be stress-related metabolites [14–16].

In this study, interesting contents of furanocoumarins were obtained in the callus cultures and proved the possibility to influence the efficacy of their accumulation by changing the nature and concentration of phytohormones in the medium. This allows to propose *Pastinaca sativa* callus cultures as a convenient model to study the biosynthesis of furanocoumarins and also as a potential source of these compounds, particularly isopimpinellin.

3. Experimental

3.1. Establishment of in vitro culture

Callus cultures were derived from hypocotyl parts of sterile seedlings (seeds were obtained from the München-Nymphenburg Botanical Garden). Callus initiation was observed on Linsmaier-Skoog's medium (L-S) [11] with the addition of phytohormones: 2 mg/l α-naphthaleneacetic acid (NAA), 2 mg/l 6-benzylaminopurine (BAP) under constant artificial light (900 lx) at a temperature of 25 ± 2 °C.

3.2. Experimental cultures

Callus cultures were maintained on variants of solid L-S medium, containing varying quantities of phytohormones (mg/l): NAA+BAP (0.1 + 0.1, 1.0 + 1.0, 2.0 + 2.0, 3.0 + 1.0, 5.0 + 5.0, 5.0 + 10.0, 10.0 + 5.0, 10.0 + 10.0) − 8 variants, and β-indolylbutyric acid (IBA) + BAP (1.0 + 1.0, 5.0 + 5.0) − 2 variants. Shoot culture was maintained on solid L-S medium containing 2 mg/l of NAA and 2 mg/l of BAP. Callus cultures and shoot culture were conducted under light conditions and temperature detailed in p. 3.1. Callus tissues and shoots were subcultured every 6 weeks.

3.3. Extraction

Dried and ground material (approximately 1 g): callus tissue and shoots cultivated *in vitro* collected after 42 days of culture (subcultures from 10–14) as well as leaves, stems and roots of the plant growing in open air (two-year-old plants were harvested in September 1999 in Kraków, Poland) and fruits from which callus cultures were derived, were extracted with two portions (50 ml) of boiling 96% ethanol in Soxhlet's apparatus for 10 h, according to the procedure described earlier [17]. Extracts were combined, thickened and evaporated to dryness. The residue was quantitatively dissolved in 10 ml of 96% ethanol, and analysed by HPLC.

3.4. HPLC

Contents of psoralen, xanthotoxin, bergapten, isopimpinellin, imperatorin and umbelliferone were determined according to a procedure described elsewhere [17]:

HPLC apparatus: Ati Unicam, Cambridge. Pump: Crystal 200 (Ati Unicam, Cambridge). Column: Supelcosil LC-8 (4.6 mm/25 cm). Solvent system: methanol–water (1 : 1.2); in the case of imperatorin: methanol–water (2 : 1). Flow rate: 1 ml/min. Detector UV: $\lambda = 310$ nm. Standards: manufactured by Carl Roth.

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