

# ANETHUM GRAVEOLENS L. IN VITRO CULTURES – A POTENTIAL SOURCE OF BIOACTIVE METABOLITES, PHENOLIC ACIDS AND FURANOCOUMARINS

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In vitro cultures of *Anethum graveolens* (dill) were maintained on the Linsmaier and Skoog (LS) medium – three variants, and the Murashige and Skoog (MS) medium – seven variants, which contained different amounts of plant growth regulators, cytokinin (BAP) and auxin (NAA) (from 0.1 mg l<sup>-1</sup> to 3.0 mg l<sup>-1</sup>). Methanolic extracts from in vitro grown biomass were analyzed by HPLC for free phenolic acids and furanocoumarins. The total amounts of free phenolic acids on the LS medium variants were similar (35.23–38.65 mg 100 g<sup>-1</sup> DW), but higher on the MS variants, ranging from about 66 mg 100 g<sup>-1</sup> DW to 100 mg 100 g<sup>-1</sup> DW. The main metabolites were: p-hydroxybenzoic acid (max. 24.41 mg 100 g<sup>-1</sup> DW) on the LS-based media, and salicylic acid (max. 57.88 mg 100 g<sup>-1</sup> DW) and p-hydroxybenzoic acid (max. 36.27 mg 100 g<sup>-1</sup> DW) on the MS-based media. The total amounts of furanocoumarins were lower, as they did not exceed 8.5 mg 100 g<sup>-1</sup> DW on the LS media and 25 mg 100 g<sup>-1</sup> DW on the MS media. The main compounds in this group were bergapten (max. 15.01 mg 100 g<sup>-1</sup> DW) and marmesin (max. 8.12 mg 100 g<sup>-1</sup> DW). The MS variant containing 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA was proposed as the best production medium for both groups of metabolites. The maximum total amounts of free phenolic acids obtained in the in vitro grown biomass were slightly higher than their amounts in the fruits of the mother plant analyzed for comparison (99.66 mg 100 g<sup>-1</sup> DW and 93.34 mg 100 g<sup>-1</sup> DW, respectively); the maximum total amounts of furanocoumarins were approximately 1.8 times higher than in the fruits (24.26 mg 100 g<sup>-1</sup> DW and 13.67 mg 100 g<sup>-1</sup> DW, respectively).

**Keywords:** Dill, Apiaceae, psoralens, plant growth regulators, secondary metabolites, HPLC analysis

## INTRODUCTION

*Anethum graveolens* L. (Eng. dill), an Apiaceae plant species native to different regions of Asia (Iran, India, the Caucasus), is cultivated as a medicinal plant and spice worldwide, including Europe and North America.

Fruits of *A. graveolens* are a rich source of different groups of metabolites, e.g. volatile oil, coumarins, flavonoids, phenolic acids, fatty oil and minerals (Heywood, 1971). Since 2007, *Anethi fructus* monograph has been included in the International Pharmacopoeia, edited by WHO (2007). Extracts from flowers have been found to contain chlorogenic acid, some flavonoids, including myricetin, and proanthocyanidins, and other phenolic compounds responsible for antioxidant ability (Shyu et al., 2009). The composition of steam-dis-

tilled essential oil of dill has also been studied (Callan et al., 2007). Fungitoxic peptides have been isolated from seed extracts (Yili et al., 2006).

Biotechnological studies of this species to date have focused on micropropagation protocols (Jana and Shekhawat 2011; 2012), ability to accumulate secondary metabolites, including flavonoids, phytosterols and coumarins, in different types of in vitro cultures (Ekiert, 2000). Most of the latest studies have been devoted to the possibility of producing essential oil in untransformed cultures as well as in hairy root (transformed) cultures (Everitt and Lockwood, 1995; Faria et al., 2009; Santos et al., 2002).

In vitro cultures of *A. graveolens* have not yet been tested for the ability to accumulate phenolic

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**Abbreviations:** BAP – 6-Benzylaminopurine, DW – Dry weight, HPLC – High-performance liquid chromatography, LS – Linsmaier and Skoog, NAA – 1-Naphthaleneacetic acid, MS – Murashige and Skoog

acids. This group of plant metabolites has highly valuable therapeutic qualities, such as antioxidative, anti-inflammatory, immunostimulating and anti-cancer (Ekiert and Czygan, 2007; Wen et al., 2003).

Several biotechnological studies, including those in our laboratory, have shown that phenolic acids can be produced under in vitro culture conditions in amounts that are of interest from a practical point of view. These successes include not only the production of rosmarinic acid (Ekiert and Czygan, 2007; Ekiert et al., 2013; Makri and Kintzios, 2004), but also of protocatechuic, p-coumaric, p-hydroxybenzoic, salicylic and other acids (Szopa et al., 2012, 2013; Szopa and Ekiert, 2012; 2014).

The aim of the presented study was to investigate the accumulation of free phenolic acids in in vitro cultures of *A. graveolens*. The study was conducted in two stages. In the first stage, the cultures were maintained on three variants of the Linsmaier and Skoog medium (1965) with different concentrations of plant growth regulators, cytokinin (6-benzylaminopurine, BAP) and auxin (1-naphthaleneacetic acid, NAA), in a concentration range from 0.1 to 2.0 mg l<sup>-1</sup>. The methanolic extracts from the biomass grown in vitro were analyzed by HPLC (Tian et al., 2005) for eleven free phenolic acids, cinnamic and benzoic acid derivatives, and depsides.

In order to obtain possibly higher amounts of this group of metabolites, the second stage of the study, involved maintaining cultures on seven variants of the Murashige and Skoog medium (1962) containing different amounts of BAP and NAA in a concentration range from 0.1 to 3.0 mg l<sup>-1</sup>. The study also included analysis for linear furanocoumarins and their biogenetic precursor – umbelliferone (seven compounds). This group of secondary metabolites has common initial stages of biogenesis with phenolic acids pathways (the shikimic acid pathway) (Ekiert and Czygan, 2007; Vanisree and Tsay, 2004). The therapeutic attractiveness of this group of metabolites (including photosensitivity, antiproliferative activity, calcium and potassium channels blocking and other activities) was also taken into consideration (Bohuslavizki et al., 1994; Ekiert and Czygan, 2007).

An incentive for undertaking research on the accumulation of furanocoumarins was also provided by the successful production of these metabolites in in vitro cultures of selected plant species, including our laboratory studies with the species Apiaceae and Rutaceae (Ekiert, 1993; 2000; Szopa et al., 2012).

For comparison purposes, extracts from the fruits of the mother plant from which the in vitro cultures were derived were also analyzed for the amounts of the two groups of metabolites.

## MATERIALS AND METHODS

### PLANT MATERIAL

Fruits of *A. graveolens* were obtained from plants growing in the Botanical Garden of the Centre for Biological Diversity Conservation of the Polish Academy of Sciences (Warsaw, Poland).

### ESTABLISHMENT OF IN VITRO CULTURES

The in vitro cultures of *A. graveolens* L. were established from hypocotyls of seedlings obtained from the fruits of mother plant growing in the Botanical Garden of the Centre for Biological Diversity Conservation of the Polish Academy of Sciences (Warsaw, Poland). The fruits were sterilized with 0.1% NaOCl for 10 min. and, after being rinsed in sterile double-distilled water, they were maintained on Murashige and Skoog (MS) (1962) solid medium supplemented with 30 g l<sup>-1</sup> of sucrose and plant growth regulators: 1 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA. After 4 weeks callus cultures were obtained. The cultures were cultivated under constant artificial light (4 W/m<sup>2</sup>, LF-40 W lamp, daylight, Piła) at 25 ± 2°C.

### EXPERIMENTAL IN VITRO CULTURES

In the first stage, experimental in vitro cultures were maintained on three solid variants of the LS (1965) medium (Fig. 1 and 3), and later on seven solid variants of the MS medium (Fig. 2 and 4) with different concentrations of plant growth regulators: BAP (0.1–3.0 mg l<sup>-1</sup>) and NAA (0.1–2.0 mg l<sup>-1</sup>), with standard amount of sucrose – 30 g l<sup>-1</sup>. The cultures were grown under the above-mentioned light conditions for four weeks (three series).

### EXTRACTION

After 4-week growth cycles (three series), the lyophilized biomass from in vitro cultures was collected (1.0 g) and subjected to extraction, which was carried out twice with boiling methanol (50 ml) under a reflux condenser for 3 h. The extracts were then combined, condensed and evaporated to dryness. The resulting residue was quantitatively dissolved in 2 ml of methanol and analyzed by HPLC. Samples from each series were analyzed individually, and the results are presented as the mean value with standard deviation (SD).

### HPLC ANALYSIS

The methanolic extracts from the biomass were analyzed for eleven phenolic acids and cinnamic acid as

one group, and six linear furanocoumarins and umbelliferone as another group, using RP-HPLC methods according to Tian et al. (2005) and those of Ekiert and Gomółka (1999), respectively.

Separation of phenolic acids was performed using a Kinetex™ C-18 analytical column (150 x 4.6 mm, 2.6 µm) at 25°C. The mobile phase consisted of 0.1% trifluoroacetic acid (A) and acetonitrile (B) at a flow rate of 1.0 ml min<sup>-1</sup> (gradient program); injection volume: 5 µl. Gradient elution was as follows: 0 min, 5% solvent B; 5 min, 9% solvent B; 15 min, 9% solvent B; 22 min, 11% solvent B; and 38 min, 18% solvent B. Detection wavelength was set at 254 nm. Quantification was made by comparison with standards: caffeic, chlorogenic, cinnamic, protocatechuic, rosmarinic, salicylic, sinapic, and syringic acids from Sigma; p-coumaric, ferulic, p-hydroxybenzoic, and vanillic acids from Fluka.

Separation of coumarins was conducted in a LiChrospher 100 RP-18 (200 x 4 mm) analytical column at 25°C. The mobile phase consisted of methanol:water (1:1.5 v/v) (A) and methanol (B) at a flow rate of 1.0 ml min<sup>-1</sup> (gradient program); injection volume: 10 µl. Gradient elution was as follows: 10 min, 0% solvent B; 20 min, 20% solvent B; 40 min, 50% solvent B; 41 min, 52% solvent B; 42 min, 55% solvent B; 43 min, 60% solvent B; 44 min, 70% solvent B; and 50 min, 90% solvent B. Detection wavelength was set at 310 nm. Quantification was made by comparison with standards: bergapten, imperatorin, psoralen, umbelliferone, xanthotoxin from Roth, and isopimpinellin, marmesin from Institute of Pharmacology, Polish Academy of Sciences, Kraków (Poland).

## RESULTS

### ESTABLISHMENT OF IN VITRO CULTURES AND INCREASES IN THE BIOMASS

In vitro cultures of *Anethum graveolens* were initiated from hypocotylous parts of seedlings obtained from the fruits of plant growing in vivo. The solid agar MS medium supplemented with plant growth regulators, 1 mg l<sup>-1</sup> BAP and 0.5 mg l<sup>-1</sup> NAA, was used as an initiation medium. Throughout subculturing, the cultures developed as callus cultures independently of the tested composition of growth regulators.

Dry biomass from callus cultivated on three variants of the LS medium increased from 4.9- to 8.7-fold over 4-week growth cycles. The highest increments were confirmed on the LS variant containing 2.0 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA.

Dry biomass from the experimental cultures maintained on seven variants of the MS medium varied greatly and increased from 4.9- to 12.9-fold

over 4-week growth cycles. The highest increases in biomass, over 12-fold, were obtained on the media containing 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA and 2.0 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA.

### ACCUMULATION OF PHENOLIC ACIDS AND CINNAMIC ACID

Out of the twelve compounds which we analyzed, the methanolic extracts from the biomass of cultures grown on three tested variants of the LS medium were found to contain three free phenolic acids: p-hydroxybenzoic acid, salicylic acid and vanillic acid; the presence of caffeic, chlorogenic, p-coumaric, ferulic, protocatechuic, rosmarinic, sinapic, syringic and cinnamic acids was not detected in any of the extracts.

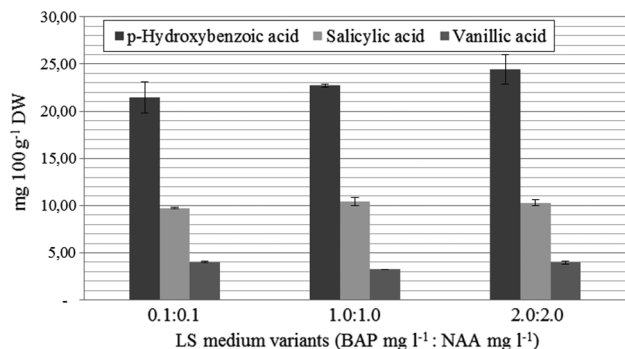
The amounts of the individual compounds and their total amounts were similar on the tested variants of the LS medium. The total amounts varied slightly from 35.23 to 38.65 mg 100 g<sup>-1</sup> DW. The maximum content was confirmed on the medium containing 2.0 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA.

The largest amounts accumulated in the in vitro grown biomass were those of p-hydroxybenzoic acid (21.47–24.41 mg 100 g<sup>-1</sup> DW). Salicylic acid was found at a level almost twice as low (9.74–10.42 mg 100 g<sup>-1</sup> DW). Vanillic acid content did not exceed 4.1 mg 100 g<sup>-1</sup> DW (Fig. 1).

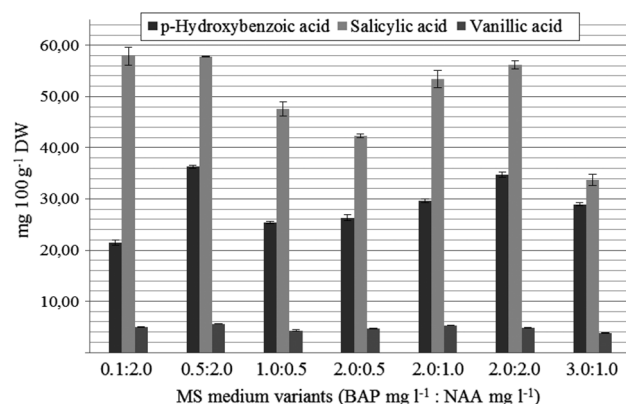
The extracts from the biomass grown on seven variants of the MS medium tested within a broader range of BAP and NAA concentrations were also found to contain three free phenolic acids, the same ones that were present in the extracts from the biomass grown on the variants of the LS medium, i.e. p-hydroxybenzoic acid, salicylic acid and vanillic acid.

The amounts of the individual compounds as well as their total amounts varied depending on the concentration of the growth regulators in the culture media. The total phenolic acids content ranged from 66.48 to 99.66 mg 100 g<sup>-1</sup> DW. The highest amounts, of more than 95 mg 100 g<sup>-1</sup> DW, were found on two MS variants, i.e. those containing 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA, and 2.0 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA.

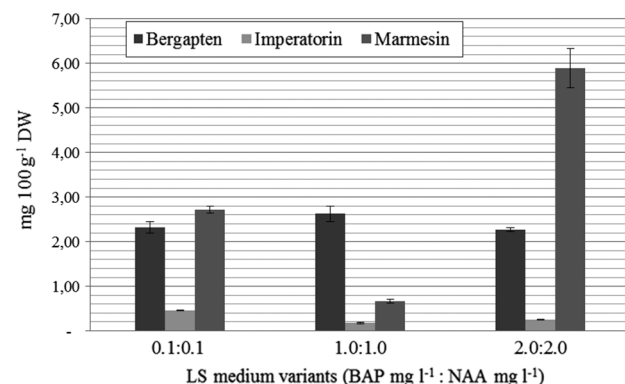
The main metabolite accumulated on the variants of the MS medium was salicylic acid. p-Hydroxybenzoic acid was accumulated in smaller quantities. The lowest amounts were those of vanillic acid. The amounts of salicylic acid varied considerably, ranging from 33.71 to 57.88 mg 100 g<sup>-1</sup> DW. High amounts of this compound, exceeding 50 mg 100 g<sup>-1</sup> DW, were found on as many as 4 variants of the MS medium, with the highest, almost identical, amounts on the media containing 0.1 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA, 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA, and 2.0 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA.



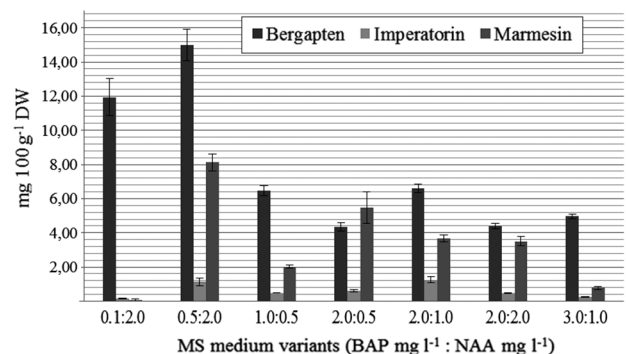
**Fig. 1.** Contents (mg 100 g<sup>-1</sup> DW) of free phenolic acids in biomass extracts from callus cultures of *A. graveolens* cultivated on LS medium variants (BAP mg l<sup>-1</sup> : NAA mg l<sup>-1</sup>). The values are means of three experiments ± SD



**Fig. 2.** Contents (mg 100 g<sup>-1</sup> DW) of free phenolic acids in biomass extracts from callus cultures of *A. graveolens* cultivated on MS medium variants (BAP mg l<sup>-1</sup> : NAA mg l<sup>-1</sup>). The values are means of three experiments ± SD



**Fig. 3.** Contents (mg 100 g<sup>-1</sup> DW) of coumarins in biomass extracts from callus cultures of *A. graveolens* cultivated on LS medium variants (BAP mg l<sup>-1</sup> : NAA mg l<sup>-1</sup>). The values are means of three experiments ± SD



**Fig. 4.** Contents (mg 100 g<sup>-1</sup> DW) of coumarins in biomass extracts from callus cultures of *A. graveolens* cultivated on MS medium variants (BAP mg l<sup>-1</sup> : NAA mg l<sup>-1</sup>). The values are means of three experiments ± SD

The amounts of p-hydroxybenzoic acid were also high and varied, ranging from 21.45 to 36.27 mg 100 g<sup>-1</sup> DW. The highest amounts of 35–37 mg 100 g<sup>-1</sup> DW were found on the media containing 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA, and 2.0 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA.

The quantities of vanillic acid were of a different order; they did not exceed 6.0 mg 100 g<sup>-1</sup> DW and ranged from 3.82 to 5.63 mg 100 g<sup>-1</sup> DW. The highest content was obtained on the medium containing 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA (Fig. 2).

#### ACCUMULATION OF FURANOCOUMARINS AND UMBELLIFERONE

Out of the seven compounds which we analyzed, the extracts from the biomass grown on three tested variants of the LS medium were found to contain three

furanocoumarins – marmesin, bergapten and imperatorin. None of the extracts contained umbelliferone, psoralen, xanthotoxin, or isopimpinellin.

The amounts of the individual compounds and their total amounts varied depending on the LS-based medium. The total amounts of the three metabolites detected were low, and ranged from 3.47 to 8.41 mg 100 g<sup>-1</sup> DW. The maximum content was found on the LS medium supplemented with 2.0 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA. The amounts of marmesin were particularly varied, ranging from 0.67 to 5.89 mg 100 g<sup>-1</sup> DW. The highest amount of it was confirmed on the LS variant containing cytokinin and auxin at 2.0 mg l<sup>-1</sup> each. The quantities of bergapten were similar, varied little, ranging from 2.27 to 2.63 mg 100 g<sup>-1</sup> DW. The maximum amount of imperatorin did not exceed 0.5 mg 100 g<sup>-1</sup> DW (Fig. 3).



TABLE 1. Contents (mg 100 g<sup>-1</sup> DW ± SD) of free phenolic acids in fruits of mother plant and their maximal contents in in vitro cultures.

Phenolic acids	Plant material	
	Callus cultures	Fruits
p-Hydroxybenzoic acid	26.27 ± 0.54*	11.40 ± 0.50
Salicylic acid	57.88 ± 1.76*	69.99 ± 4.23
Vanillic acid	5.63 ± 0.06**	11.96 ± 0.06
Total contents	99.66 ± 0.41*	93.34 ± 4.79

\*MS medium variant with 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA  
 \*\*MS medium variant with 2.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> NAA

TABLE 2. Contents (mg 100 g<sup>-1</sup> DW ± SD) of coumarins in fruits of mother plant and their maximal contents in in vitro cultures.

Coumarins	Plant material	
	Callus cultures	Fruits
Bergapten	15.01 ± 0.92*	7.94 ± 0.29
Imperatorin	1.26 ± 0.18**	1.17 ± 0.19
Marmesin	8.12 ± 0.50*	4.56 ± 0.67
Total contents	24.26 ± 1.65*	13.67 ± 1.15

\*MS medium variant with 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA  
 \*\*MS medium variant with 2.0 mg l<sup>-1</sup> BAP and 1.0 mg l<sup>-1</sup> NAA

In terms of qualitative composition, the extracts from the biomass grown on seven variants of the MS medium contained the same coumarin compounds as those obtained on the LS variants. The total amounts of the three metabolites – marmesin, bergapten and imperatorin, varied within a wide range, from 5.99 to 24.26 mg 100 g<sup>-1</sup> DW. The maximum content was found on the MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA.

The largest amounts accumulated in the biomass were those of bergapten. They varied greatly, ranging from 4.35 to 15.01 mg 100 g<sup>-1</sup> DW. The highest amounts of this compound were found on the media containing 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA and 0.1 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA.

The quantities of marmesin varied enormously, ranging from 0.08 to 8.12 mg 100 g<sup>-1</sup> DW. The maximum content was found on the medium containing 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA. The amounts of imperatorin were very low, only up to 1.26 mg 100 g<sup>-1</sup> DW, on all the tested variants of the MS medium (Fig. 4).

#### MOTHER PLANT MATERIAL

The qualitative composition of the extracts from the fruits of the mother plant was found to be identical to that of the extracts from the in vitro grown biomass. Quantitatively, the predominant metabolite was salicylic acid (about 70 mg 100 g<sup>-1</sup> DW); the amounts of the other two acids, p-hydroxybenzoic and vanillic acid, were almost the same, 11.40 and 11.96 mg 100 g<sup>-1</sup> DW, respectively (Table 1).

The fruits extract were confirmed to contain the three linear furanocoumarins – marmesin, bergapten and imperatorin. The main compound in this group of metabolites was bergapten (7.94 mg 100 g<sup>-1</sup> DW). The amount of marmesin was lower (4.56 mg 100 g<sup>-1</sup> DW), and that of imperatorin very low (1.17 mg 100 g<sup>-1</sup> DW). The total amount of all the furano-coumarins did not exceed 14.0 mg 100 g<sup>-1</sup> DW (Table 2).

#### DISCUSSION

The concentration of plant growth regulators in culture media has a huge impact on both the increase in biomass and the accumulation of metabolites (Ramawat and Mathur, 2007). In the present study there was a marked increase in the growth rate of *Anethum graveolens* biomass attributed to the cytokinin and auxin concentrations in LS-based media (from 4.9 to 8.7 fold). These growth increments were higher than in callus cultures of other Apiaceae species cultivated earlier in our laboratory. In *Pastinaca sativa* cultures we had obtained up to a 3-fold increase in fresh biomass growth during 6-week subcultures (Ekiert and Gomółka, 2000 b), and in cultures of *Ammi majus* there had been a 4.4-fold increase in the growth of fresh biomass during 4-week subcultures (Ekiert and Gomółka, 2000 a). By comparison, in callus cultures of *Aronia melanocarpa* (Rosaceae) grown on different variants of the LS medium, the increases in dry biomass fluctuated within the range from 2.0 to 4.8 times (Szopa et al., 2013). In all those experiments, the same plant growth regulators were added to the LS medium.

There was a much greater variation in the growth of *A. graveolens* biomass on MS-based media (from 4.9- to 12.9-fold). The resulting maximum increases in dry biomass, nearly 13-fold over 4-week growth cycles, were very high. In our studies with in vitro cultures of plant species from different taxa we had usually obtained lower increases in dry biomass over 4-week growth cycles. In *A. melanocarpa* callus cultures cultivated on MS media variants, the increases in the biomass over 4-week growth cycles had been lower (3.3- to 5.0-fold) (Szopa and Ekiert, 2014). We had obtained slightly larger increases (about 7.5-fold) in callus cultures of *Schisandra chinensis* cultivated on two variants of the MS medium (Szopa and Ekiert, 2011; 2012; 2015; Szopa et al., 2012).

The resulting maximum increases in dry biomass in *A. graveolens* were obtained on MS-based media in which the cytokinin to auxin ratio was 1:4 or 1:1. Those two variants of the MS medium, containing 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA and 2.0 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA, can be proposed as good 'growth media'. The same media were the best 'growth media' for *A. melanocarpa* callus cultures (Szopa and Ekiert, 2014), whereas the later variant was the best for increasing biomass growth in *S. chinensis* callus culture (Szopa and Ekiert, 2011; 2012; 2015; Szopa et al., 2012).

The effect of growth regulators on the accumulation of different groups of secondary metabolites is a well-known phenomenon (Ramawat and Mathur 2007). It has been documented for different plant growth regulators which influence the production of various groups of secondary metabolites, e.g. anthraquinones (hypericin, pseudohypericin), phloroglucine derivate (hyperforin) in *Hypericum* sp. shoot cultures (Coste et al., 2011), flavonoid (rutin) in *Morus alba* in vitro cultures (Lee et al., 2011), terpenoids in *Camellia sinensis* suspension cultures (Grover et al., 2012), and alkaloid (galanthamine) production in in vitro cultures of three Amaryllidaceae species (El Tahchy et al., 2011). We had documented this relationship in our earlier studies on the accumulation of linear furanocoumarins in *A. majus* (Ekiert, 1993; Ekiert and Gomółka, 2000 a) and *P. sativa* cultures (Ekiert and Gomółka, 2000 b), and on the accumulation of lignans in *S. chinensis* cultures (Szopa and Ekiert, 2011; 2013; 2015). We had also observed the dependence of free phenolic acid accumulation in *Ruta graveolens*, *R. graveolens* ssp. *divaricata*, *A. melanocarpa* and *S. chinensis* in vitro cultures on the composition of growth regulators (Ekiert et al., 2009; Szopa and Ekiert, 2012, 2014, 2015; Szopa et al., 2012; 2013). Now we have demonstrated empirically the significance of the concentration of the tested plant growth regulators in culture media on the accumulation of free phenolic acids and furanocoumarins in *A. graveolens* in vitro cultures.

In all the examined extracts from in vitro cultures of *A. graveolens* we obtained an identical qualitative composition of free phenolic acids. In all the extracts analyzed, we confirmed the presence of three compounds: p-hydroxybenzoic acid, salicylic acid and vanillic acid. In quantitative terms, the amounts of these compounds from LS-based media were almost identical. In the three tested variants of the LS medium, the absolute concentrations of BAP and NAA increased from 0.1 mg l<sup>-1</sup> to 2.0 mg l<sup>-1</sup>, but the cytokinin and auxin ratio was always 1:1. Similarly, in cultures of *R. graveolens* ssp. *divaricata* conducted on three variants of the LS medium containing BAP and NAA in the amounts of: 0.1 and 0.1, 1.0 and 1.0, and 2.0 and 2.0 mg l<sup>-1</sup>, i.e. at the

same 1:1 ratio, we had found almost the same levels of phenolic acids (Ekiert et al., 2014).

In the case of the tested variants of the MS medium, the cytokinin/auxin ratio varied considerably and the effect of plant growth regulators on the accumulation of the analyzed metabolites was more evident. Depending on the variant of the MS medium, we found changes from 1.47- to 1.72-fold in the amounts of the three phenolic acids being determined: p-hydroxybenzoic acid, salicylic acid and vanillic acid.

The total amounts of free phenolic acids ranged from 66.48 to 99.66 (a 1.50-times increase) on the tested variants of the MS medium. The highest amounts of 99.66 mg 100 g<sup>-1</sup> DW and 95.77 mg 100 g<sup>-1</sup> DW were obtained on the media containing 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA, and 2.0 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA, respectively. These variants of the MS medium can be nominated as 'productive media'. The same variants were also the most favourable to increasing the growth in biomass. These media can therefore be proposed as universal, 'growth' and 'productive', media. The medium containing 2.0 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA was a good growth-promoting medium for *A. melanocarpa* and *S. chinensis* callus cultures (Szopa and Ekiert, 2012; 2013; 2014; 2015).

Such a situation is relatively rare. Plant cultures in vitro are usually carried out in two stages, first on a medium that favours the growth in biomass, then on a medium with a different composition favourable to the production of metabolites. However, in our previous studies, we had proposed universal, both 'productive' and 'growth promoting', media for callus cultures of both *A. melanocarpa* and *S. chinensis* (Szopa and Ekiert, 2012; 2013; 2014; 2015).

A comparison of the amounts of individual phenolic acids on LS- and MS-based media with identical concentrations of BAP and NAA (2.0 mg l<sup>-1</sup> each) indicates that it is possible to obtain from 1.21 to 5.47 times higher amounts of these metabolites on the MS medium than it is on the LS medium. Also, their total amount is almost 2.5 times higher on the MS medium. It would appear that the richer chemical composition of the MS medium, being a richer source of vitamins, stimulates the various enzymatic reactions. We had obtained different results in callus cultures of *A. melanocarpa* – the total amounts of the phenolic acids determined in the biomass cultivated on two identical variants of MS and LS media were almost the same. Only on one variant, containing 3 mg l<sup>-1</sup> BAP and 1 mg l<sup>-1</sup> NAA, the total content was 1.22 times higher on the MS-based than on the LS-based medium (Szopa and Ekiert, 2013; 2014).

The maximum total phenolic acid content in the biomass from in vitro cultures was slightly higher than in the fruits of the mother plant (93.34 mg 100 g<sup>-1</sup> DW).

Considering the low degree of differentiation of the in vitro cultured biomass (undifferentiating callus), this result should be regarded as a success. In addition, the very high increases in biomass indicate potential applicability of the results.

The three phenolic acids whose presence was confirmed in the biomass extracts, and also in the fruit extracts, are derivatives of benzoic acid. In none of the extracts was the presence of cinnamic acid derivatives detected. It appears that the paths of the biogenesis of benzoic acid derivatives are dominant both in the tested plant growing in vivo and in the biomass cultured in vitro.

All the extracts analyzed were shown to contain the same three linear furanocoumarins – marmesin, bergapten and imperatorin. In the case of the tested LS media, in which the cytokinin/auxin ratio was always 1:1, there were large differences in the amounts of the analyzed coumarins. The amounts of individual metabolites in this group increased 8.79-, 1.16- and 2.50-fold, respectively. The total amount of these compounds increased 2.5-fold. We had demonstrated similarly large variations in the amounts of coumarins in callus cultures of Apiaceae species growing on different variants of the LS medium enriched with BAP and NAA. In *A. majus* cultures, the total amount of the compounds was from 40.95 to 871.05 mg 100 g<sup>-1</sup> DW (Ekiert and Gomółka, 2000 a), and in *P. sativa* cultures – from 115.70 to 408.50 mg 100 g<sup>-1</sup> DW (Ekiert and Gomółka, 2000 b). However, Tirillini et al. (1998) were unable to document the dependence of the accumulation of coumarins on the concentration of plant growth regulators in their experiment with callus cultures of *Heracleum sphondylium* ssp. *sphondylium*, possibly due to a narrow concentration range of 2.4-D and kinetin (0.1 to 1.0 mg l<sup>-1</sup>). Furthermore, in comparison with our in vitro cultures, these authors obtained considerably lower amounts of four coumarins: bergapten, imperatorin, isopimpinellin and xanthotoxin (0.7 to 5.1 µg g<sup>-1</sup>).

On the tested variants of the MS medium, the amounts of these compounds increased within an even broader range, from 3.45 to 101.50 times, and the total content increased 4.05 times.

The maximum total amounts obtained in the biomass grown on the LS- and MS-based media, equal to 8.41 and 24.26 mg 100 g<sup>-1</sup> DW, respectively, were relatively low. The highest content was obtained on the MS medium supplemented with 0.5 mg l<sup>-1</sup> BAP and 2.0 mg l<sup>-1</sup> NAA, i.e. the same medium that favoured the production of phenolic acids and increase in biomass.

The maximum total amount of furanocoumarins in the biomass from the in vitro cultures was 1.77 times higher than in the fruits analyzed for comparison (13.67 mg 100 g<sup>-1</sup> DW).

Higher levels of coumarins can be expected after using elicitors. Reports from other research centres have demonstrated stimulation of the biosynthesis of these stress-related metabolites by means of different elicitors, for example in in vitro cultures of *R. graveolens* (Orlita et al., 2008 a; b). The current literature contains several reports describing the significance of elicitors for the production of coumarins, including linear furanocoumarins in Apiaceae and Rutaceae species (Sidwa-Gorycka et al., 2003).

We propose our in vitro cultures of *A. graveolens* as a good model for studying the accumulation of the two groups of secondary metabolites, especially free phenolic acids, as well as a potential biotechnological source for obtaining hydroxybenzoic acids: p-hydroxybenzoic acid and o-hydroxybenzoic acid (salicylic acid).

The both hydroxybenzoic acids exhibit biological activities of interest. p-Hydroxybenzoic acid shows antimicrobial, antifungal, antisickling and estrogenic activities. o-Hydroxybenzoic acid (salicylic acid) has anti-inflammatory, antiseptic, antifungal, antipyretic, analgesic and keratolytic properties.

#### AUTHORS' CONTRIBUTIONS

A. Szopa compiled the data, performed the chemical and statistical analysis. H. Ekiert conceived the idea of the paper and prepared the manuscript. A. Szopa prepared the graphical version of the manuscript. The authors declare that there are no conflicts of interests.

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