Chair and Department of Pharmaceutical Botany[1], Collegium Medicum, Jagiellonian University, Kraków, Poland, Lehrstuhl für Pharmazeutische Biologie[2], Julius-von-Sachs-Institut für Biowissenschaften der Universität Würzburg, Germany

Accumulation of biologically active furanocoumarins in agitated cultures of *Ruta graveolens* L. and *Ruta graveolens* ssp. *divaricata* (Tenore) Gams

H. Ekiert[1], F.-Ch. Czygan[2]

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Dr Halina Ekiert, Chair and Department of Pharmaceutical Botany, Collegium Medicum, Jagiellonian University, 9 Medyczna Street, 30-688 Kraków, Poland
mfekiert@cyf-kr.edu.pl

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This study was designed to investigate the dynamics of accumulation of linear furanocoumarins (psoralen, bergapten, xanthotoxin, isopimpinellin, imperatorin) and their biogenetic precursor, umbelliferone, in agitated cultures of *Ruta graveolens* L. and *Ruta graveolens* ssp. *divaricata* (Tenore) Gams during 6-week growth cycles. The metabolites under study were almost exclusively accumulated in the cultured biomass. The total content of all metabolites increased 4.8- and 2.0-fold, in *R. graveolens* and *R. graveolens* ssp. *divaricata* cultures, respectively. Xanthotoxin and bergapten, which are the most important therapeutic compounds, were the dominating metabolites in cultures of both plants. The maximum content of xanthotoxin (25.0 mg/100 g dry wt.) and bergapten (18.4 mg/100 g dry wt) and the maximum content of all metabolites (64.0 mg/100 g dry wt) in *R. graveolens* ssp. *divaricata* callus obtained on the 35th culture day were relatively low. However, maximum contents of xanthotoxin (136.8 mg/100 g dry wt), bergapten (210.4 mg/100 g dry wt.) and isopimpinellin (96.7 mg/100 g dry wt), and total content of all metabolites in *R. graveolens* shoots (520.8 mg/100 g dry wt) obtained on the 42nd culture day are interesting from a practical point of view.

1. Introduction

In vitro cultures of medicinal plants can be an alternative source of biologically active secondary metabolites. Furanocoumarins, psoralen derivatives, and particularly bergapten and xanthotoxin are a group of such metabolites, due to their antiproliferative and photosensitizing properties (Bethea et al. 1999). In addition, some furanocoumarins were shown to act as calcium channel blockers (Härmälä et al. 1992). This group of metabolites has also been studied for efficiency in neurological disorders like e.g. multiple sclerosis (Bohuslavizki et al. 1994; Koppenhöfer 1995).

The ability of *Ruta graveolens* L. (Rutaceae) callus, cell suspension, shoot-differentiating and shoot cultures to produce linear furanocoumarins was reported previously (Reinhard et al. 1971; Petit-Paly et al. 1986). We have established a high-producing shoot culture of this plant species. The shoots maintained in stationary liquid phase were found to accumulate very high amounts of xanthotoxin (332.0 mg/100 g dry wt), bergapten (324.0 mg/100 g dry wt) and isopimpinellin (117.0 mg/100 g dry wt). The total content of furanocoumarins was equal to or higher than in plants growing in open air (Ekiert and Gomółka 1999; Ekiert et al. 2001a).

We studied also stationary liquid cultures of *Ruta graveolens* ssp. *divaricata* (Tenore) Gams. This plant is considered by Hegi (1965) and Hoppe (1975) a subspecies of *Ruta graveolens* L. In shoot-differentiating callus from stationary liquid culture, we also obtained considerable amounts of xanthotoxin (112.0 mg/100 g dry wt). The contents of bergapten (76.0 mg/100 g dry wt) was higher than in the plants growing in open air. Furthermore, we noted that isopimpinellin was accumulated only in the cultured biomass (max. 84.0 mg/100 g dry wt). Our results are the first report demonstrating furanocoumarin production in an *in vitro* culture of this subspecies (Ekiert et al. 2005).

We propose the stationary cultures of both *R. graveolens* and *R. graveolens* ssp. *divaricata* as a good source of the aforementioned three therapeutically important furanocoumarins – xanthotoxin, bergapten and isopimpinellin (Ekiert et al. 2001a, 2005).

In order to meet practical requirements for biotechnological application of the procedures under examination in this study, we made an attempt to examine agitated cultures of both plants as a preparatory step before possible culture in a fermenter. Preliminary experiments with agitated cultures of both plants were successful. In those studies, LS medium according to Linsmaier and Skoog (1965) containing 0.1 mg/l NAA and 0.1 mg/l BAP was selected as beneficial for accumulation of the investigated furanocoumarins (Ekiert et al. 2001b). We now present detailed studies into the dynamics of accumulation of psoralen, bergapten, xanthotoxin, isopimpinellin, imperatorin, and their biogenetic precursor, umbelliferone, in agitated cultures of *R. graveolens* and *R. graveolens* ssp. *divaricata* maintained on this variant of LS medium during 6-week growth cycles.

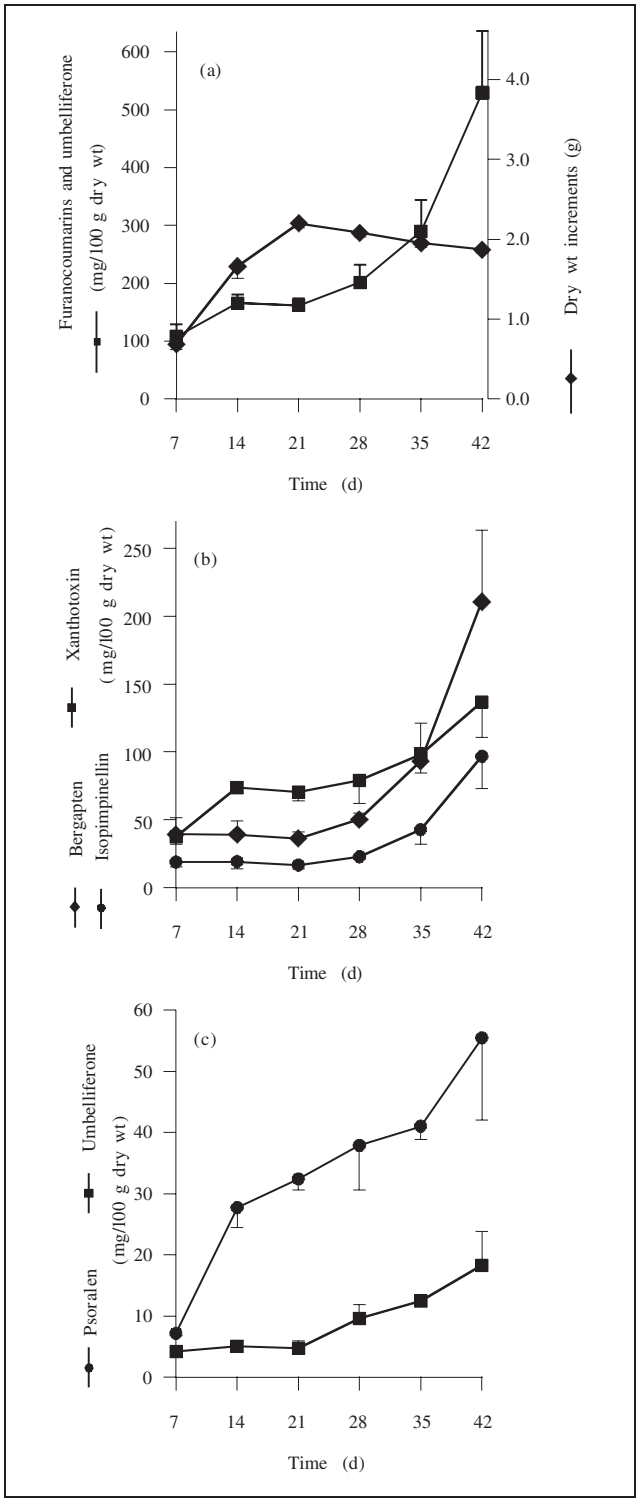

Fig. 1: The increase in dry biomass and total production of furanocoumarins and umbelliferone (a) and production of individual furanocoumarins (b), (c) in shoots of *Ruta graveolens* L. during 6-week growth cycles. The values are the means ± SEM of three experiments. Due to the low content of imperatorin (max. 3.2 mg/100 g dry wt), this metabolite has not been included in the figures (b), (c)

Table: Maximum amounts of the analyzed metabolites in the biomass from agitated cultures of *R. graveolens* L. and *R. graveolens* ssp. *divaricata* (Tenore) Gams

Metabolites	*R. graveolens*		*R. graveolens* ssp. *divaricata*	
	mg/100 g dry wt.	Day of growth cycle	mg/100 g dry wt	Day of growth cycle
Psoralen	55.5	42	1.5	28
Bergapten	210.4	42	18.4	35
Xanthotoxin	136.8	42	25.0	35
Isopimpinellin	96.7	42	6.4	35
Imperatorin	3.2	42	4.4	28
Umbelliferone	18.2	42	10.5	28
All metabolites	520.8	42	64.0	35

2. Investigations, results and discussion

2.1. Cultures of Ruta graveolens

An increase in biomass, 4.4-fold for fresh and 3.2-fold for dry biomass, was observed during 6-week growth cycles. Stationary phase of the culture growth began on culture day 21, which coincided with the beginning of intensive accumulation of the tested metabolites (Fig. 1a). All investigated metabolites were accumulated almost exclusively in shoots (99.4%), while only negligible amounts were found in the media.

Contents of the metabolites changed markedly during 6-week growth cycles, ranging from 39.4 to 210.4 mg/100 g dry wt for bergapten, from 37.5 to 136.8 mg/100 g dry wt for xanthotoxin, from 19.0 to 96.7 mg/100 g dry wt for isopimpinellin and from 7.2 to 55.5 mg/100 g dry wt for psoralen. Imperatorin amounts were markedly lower (max. 3.2 mg/100 g dry wt). Umbelliferone was accumulated at quantities from 4.2 to 18.2 mg/100 g dry wt. Maximum contents of all individual furanocoumarins and umbelliferone were observed on the 42nd culture day (Figs. 1b, c, Table).

The total content of all examined compounds increased from 107.9 to 520.8 mg/100g dry wt, reaching its maximum on culture day 42 (Fig. 1a, Table).

In tested agitated culture, the therapeutically important compounds – bergapten, xanthotoxin and isopimpinellin were accumulated in the largest quantities. Their maximum contents, amounting to 210.4, 136.8 and 96.7 mg/100 g dry wt, respectively, are undoubtedly interesting from a practical point of view.

The total content of the compounds was also substantial (520.8 mg/100 g dry wt) in comparison with the value in plants growing in open air, in which, depending on harvest location, it was very diverse, as measured in our earlier studies, and reached 575, 633 and 1033 mg/100 g dry wt (Ekiert and Gomółka 1999). The contents of bergapten and isopimpinellin, which we obtained in the present agitated cultures were decidedly higher (more than 5-fold and 1.2-fold, respectively) in comparison with stationary cultures maintained on LS medium with identical concentration of growth regulators. The amounts of xanthotoxin were also high and almost identical in both culture types. Total content of all metabolites was 1.5 times higher in agitated cultures under the present study (Ekiert and Moroniewicz 2003).

However, contents of bergapten, xanthotoxin and isopimpinellin were lower than in shoots of *R. graveolens* cultured *in vitro* on Gamborg medium by French research team (Massot et al. 2000) and slighter than in the rue shoots cultured by us in stationary liquid phase on LS medium supplemented with 2 mg/l NAA and 2 mg/l BAP (Ekiert et al. 2001a).

The dynamics of accumulation of the metabolites in the agitated culture is interesting. In spite of the beginning of the stationary phase of culture growth, content of the me-

tabolites constantly rose from day 21 until day 42. The biggest jump in concentrations of the compounds was noted at the end of growth cycles, between day 35 and 42 (Fig. 1). Amounts of the metabolites declined by about 14–45% on the average within the next 2 weeks (this observation is based on one culture series maintained for 8 weeks). In shoots cultured in stationary liquid phase on LS medium containing 0.1 mg/l NAA and 0.1 mg/l BAP after initial drop in the content of furanocoumarins, a rise in their amounts was observed between 14th and 21st day of growth cycle, which persisted at the same uniform level throughout the whole stationary phase until the end of a growth cycle (day 42) (Ekiert and Moroniewicz 2003). In *R. graveolens* agitated shoot culture maintained on Gamborg medium (with an addition of other growth regulators, kinetin and 2,4-D), Massot et al. (2000) demonstrated that after the initial increase in contents of the furanocoumarins-psoralen, bergapten, xanthotoxin and isopimpinellin within the first four days of culture, their concentrations markedly decreased and remained at the lower level until culture day 26 (the end of growth cycles).

Reinhard et al. (1971) demonstrated a gradual increase in rutamarin content in *R. graveolens* tissue cultures during its exponential growth phase, while Okazaki et al. (1982) noticed that intensive accumulation of scopolin and scopoletin in *Nicotiana tabacum* L. began with commencement of stationary phase.

2.2. Cultures of Ruta graveolens ssp. divaricata

In agitated culture of *R. graveolens* ssp. *divaricata*, there was a 5.4-fold and 3.9-fold increase in fresh and dry biomass, respectively, within 6-week growth cycles (Fig. 2a). Dynamics of biomass growth in *R. graveolens* ssp. *divaricata* culture was different from that in *R. graveolens* culture. The intensive growth phase lasted one week longer, from day 7 to 28, and the stationary growth phase began on day 28 (Figs. 1a, 2a). Intensive accumulation of the metabolites began on day 14, which is earlier than the onset of stationary growth phase (Fig. 2a).

98.3% of the metabolites were accumulated in the biomass. Bergapten and xanthotoxin were the dominating metabolites in biomass extracts. Their contents ranged from 7.1 to 18.4 mg/100 g dry wt and from 12.6 to 25.0 mg/100 g dry wt, respectively. Accumulation of other furanocoumarins was low (below 6.5 mg/100 g dry wt). Umbelliferone amounts were a little higher (from 4.4 to 10.5 mg/100 g dry wt).

Maximum contents of bergapten, xanthotoxin and isopimpinellin were detected on day 35 of the culture, while the highest accumulation of psoralen, imperatorin and umbelliferone was observed on day 28 (Table). After maximum had been reached, contents of all individual metabolites, particularly of bergapten and xanthotoxin dropped (Fig. 2b).

The total content of all metabolites under study increased 2-fold during 6-week growth cycles, from 31.3 to 64.0 mg/100g dry wt. The maximum total content was observed on day 35 (Fig. 2a, Table).

The obtained total amounts of the metabolites were lower than those noted in plants growing in open air and than in shoot-differentiating callus cultured in stationary liquid phase as well on LS medium containing 2 mg/l NAA and 2 mg/l BAP (Ekiert et al. 2005) as on the LS medium supplemented with identical concentrations of growth regulators as in the presently tested agitated culture (Ekiert et al., unpublished). This result can be explained by the low degree of differentiation of the tissues in agitated culture. In this culture type, shoot buds, that are characteristic of stationary liquid culture, were not formed. Moreover, we demonstrated in our earlier studies that metabolism of cells in *in vitro* culture of *R. graveolens* ssp. *divaricata* is directed towards the biosynthesis of other coumarin compounds, which competes with the biogenetic pathway leading to the formation of linear furanocoumarins, psoralen derivatives, which are the focus of our interest (Matern 1999). Rutacultin and dimethylether 3-(1′,1′-dimethylallyl-daphnetin), among other coumarin compounds, were the main, dominating fractions, which could easily be isolated from shoot-differentiating callus maintained in stationary liquid phase. Identity of these compounds was confirmed with spectral methods (EI-MS, ^{1}H NMR) (Ekiert et al., unpublished).

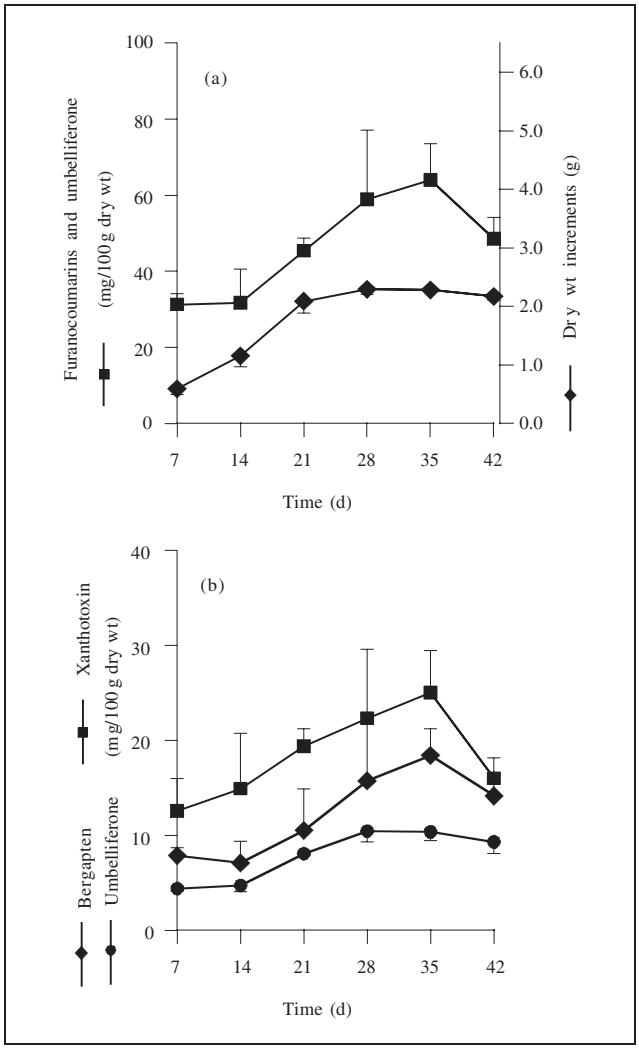

Fig. 2: The increase in dry biomass and total production of furanocoumarins and umbelliferone (a) and production of individual furanocoumarins (b) in tissues of *Ruta graveolens* ssp. *divaricata* (Tenore) Gams during 6-week growth cycles. The values are the means ± SEM of three experiments. Due to low content of psoralen, isopimpinellin and imperatorin (max. 6.4 mg/100 g dry wt), these metabolites have not been included in the figure (b)

2.3. Comparison of cultures of both plants

In summary, it was demonstrated that the tested agitated cultures of *R. graveolens* and *R. graveolens* ssp. *divaricata*,

differed in dynamics of accumulation of coumarin compounds and dynamics of biomass growth during 6-week growth cycles (Figs. 1, 2, Table).

From a biotechnological point of view, *R. graveolens* agitated shoot culture is a more interesting study object. Maximum total content of furanocoumarins and umbelliferone (520.8 mg/100 g dry wt), which was obtained in this culture, is suitable for practical applications. Maximum contents of therapeutically important metabolites (bergapten, xanthotoxin and isopimpinellin) accumulated in this culture are also high (210.4, 136.8 and 96.7 mg/100 g dry wt, respectively). We propose this culture type of *R. graveolens* shoots as a new, potential, rich source of biologically active furanocoumarins, bergapten, xanthotoxin, and isopimpinellin.

3. Experimental

3.1. Initial cultures

Initial shoots cultures of *R. graveolens* L. were maintained in stationary liquid phase in Petri dishes (for details see Ekiert and Kisiel 1997) on Linsmaier and Skoog (1965) – LS medium supplemented with growth regulators: α-naphthaleneacetic acid (NAA) – 2 mg/l, and 6-benzylaminopurine (BAP) – 2 mg/l, under artificial constant light with an intensity of 900 lx (LF – 40 W lamp, daylight, Piła), at 25 ± 2 °C.

Initial shoot-differentiating callus cultures of *R. graveolens* ssp. *divaricata* were maintained in stationary liquid phase on the same medium at the same external conditions (light, temperature) as *R. graveolens* cultures. This culture was established at the Institut für Biowissenschaften, Universität Würzburg (Germany). For details see Ekiert et al. (2005). Cultures of both plants were subcultured at about 5–6-week intervals.

3.2. Agitated cultures

Cultures of both plants were maintained on LS medium supplemented with NAA – 0.1 mg/l and BAP – 0.1 mg/l, under the same light conditions and at the same temperature as initial cultures. The cultures were maintained in Erlenmayer flasks (500 ml), containing 125 ml of the medium (initial biomass was 4 g). The flasks were shaken at a rate of 140 rpm. The cultures (3 culture series) were maintained for 6 weeks. At 1 week intervals, i.e. on day 7, 14, 21, 28, 35 and 42, biomass was separated from the medium, fresh weight was determined, and then fresh biomass was dried. The media were frozen and lyophilized. An additional culture series of *R. graveolens* was maintained for 8 weeks.

3.3. Extraction and estimation of the contents of the metabolites

Dried, powdered biomass (about 1 g), collected at 1-week intervals (after 7, 14, 21, 28, 35 and 42 days) from 3 culture series was separately extracted with boiling 96% ethanol (two portions, 10 h) in a Soxhlet apparatus according to the procedure described by Ekiert and Gomółka (1999). The extracts were combined and evaporated to dryness. The residue was quantitatively dissolved in 96% ethanol. The lyophilized media were quantitatively dissolved in 96% ethanol, as well. In these extracts and in the lyophilized media, psoralen, bergapten, xanthotoxin, isopimpinellin, imperatorin and umbelliferone were determined by HPLC. Details of the method were described earlier (Ekiert and Gomółka 1999). Briefly, the separation was performed using a Supelcosil LC-8 analytical column (4.6 mm × 25 cm), with the mobile phase: methanol/water (1 + 1.2 v/v) and (2 + 1 v/v) for imperatorin. The flow rate was 1.0 ml/min. Metabolites were detected at 310 nm. Quantities of the estimated compounds were determined by comparison with reference substances (manufactured by C. Roth).

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