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Accumulation of biologically active furanocoumarins in *Ruta graveolens* ssp. *divaricata* (Tenore) Gams *in vitro* culture

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The study was designed to investigate dynamics of accumulation of five linear furanocoumarins and umbelliferone in stationary liquid cultures of *Ruta graveolens* ssp. *divaricata* (Tenore) Gams during 6-week growth cycles. The contents of individual metabolites in biomass increased 1.8–3.5 times while their total content rose 2.3 times. Maximum contents of xanthotoxin, bergapten and isopimpinellin (112.3, 76.2 and 84.0 mg/100 g d.w., respectively) and maximum total content of all metabolites (283.4 mg/100 g d.w.), obtained on 35th culture day, are interesting from practical point of view.

1. Introduction

In the search for a good biotechnological source of biologically active linear furanocoumarins, we directed our attention to an *in vitro* culture of *Ruta graveolens* ssp. *divaricata* (Tenore) Gams (*Rutaceae*). This plant is considered by Hegi (1965) and Hoppe (1975) to be a subspecies of *Ruta graveolens* L.

Antiproliferative and photosensitising properties of linear furanocoumarins, particularly xanthotoxin, bergapten and isopimpinellin, have been applied in the treatment of skin diseases, characterised by excessive cell proliferation (e.g. psoriasis, mycosis fungoides) or in pigmentation disturbances (e.g. vitiligo, urticaria pigmentosa) (Pathak et al. 1981). In addition, some furanocoumarins were shown to act as calcium channel blockers (Härmälä et al. 1992). Moreover, bergapten was demonstrated to be a strong selective blocker of axolemmal potassium channels and is also studied in neurological affections like multiple sclerosis (Bohuslavizki et al. 1994).

Composition of the volatile oil of *Ruta graveolens* ssp. *divaricata* (Tenore) Gams was investigated earlier. Those studies showed differences in its qualitative composition in comparison with *Ruta graveolens* L. (Kubeczka, op. cit. Abou-Mandour 1982). We were interested in the contents of furanocoumarins. Beside other coumarin compounds, e.g. rutamarin and daphnoretin methyl ether, we isolated linear furanocoumarins, xanthotoxin and bergapten, which are interesting from a therapeutic point of view, from overground parts of plants growing in open air and identified them with spectral methods (^{1}H NMR, EI-MS). We also isolated xanthotoxin and bergapten from callus cultured *in vitro* (Ekiert and Kisiel, unpublished).

Hitherto existing literature data on *in vitro* culture of *Ruta graveolens* ssp. *divaricata* are scarce. They were focused on the growth of cells and tissues under different culture conditions, and on the ability of their differentiation and regeneration (Abou-Mandour 1982, Hartung and Abou-Mandour 1996).

The present paper describes the accumulation of biologically active furanocoumarins and umbelliferone in a callus culture of this subspecies maintained in a stationary liquid phase. The results of HPLC analysis of extracts from tissues cultured *in vitro* and from overground parts of plants growing in open air, analysed for comparison, are reported.

2. Investigations, results and discussion

Our *R. graveolens* ssp. *divaricata* (Tenore) Gams callus tissues cultured on Linsmaier and Skoog (1965) medium containing 2 mg/l NAA and 2 mg/l BAP exhibited certain degree of differentiation, forming sometimes buds of overground shoots.

We observed 3.7- and 3.0-fold fresh and dry biomass growth, respectively, during 6-week growth cycles. Intensive growth phase of the culture lasted culture day 7–28 (Fig.). The HPLC analysis of ethanol extracts of the cultured biomass showed that the contents of individual metabolites rose 1.8–3.5 times within 6-week subcultures, ranging from 62.8 to 112.3 mg/100 g of dry weight of biomass for xanthotoxin, from 29.4 to 76.2 mg/100 g d.w. for bergapten, from 26.1 to 84.0 mg/100 g d.w. for isopimpinellin, from 2.2 to 7.6 mg/100 g d.w. for imperatorin and from 1.7 to 5.5 mg/100 g d.w. for psoralen. The presence of umbelliferone, biogenetic precursor of furanocoumarins was detected only in few of the analysed callus extracts. The maximum contents of three quantitatively dominating metabolites: xanthotoxin, bergapten and isopimpinellin were observed on 35th culture day (Fig.). The total content of furanocoumarins and umbelliferone increased 2.3 times during 6-week subcultures, reaching its maximum (283.4 mg/100 g d.w.) on the 35th culture day (Fig.).

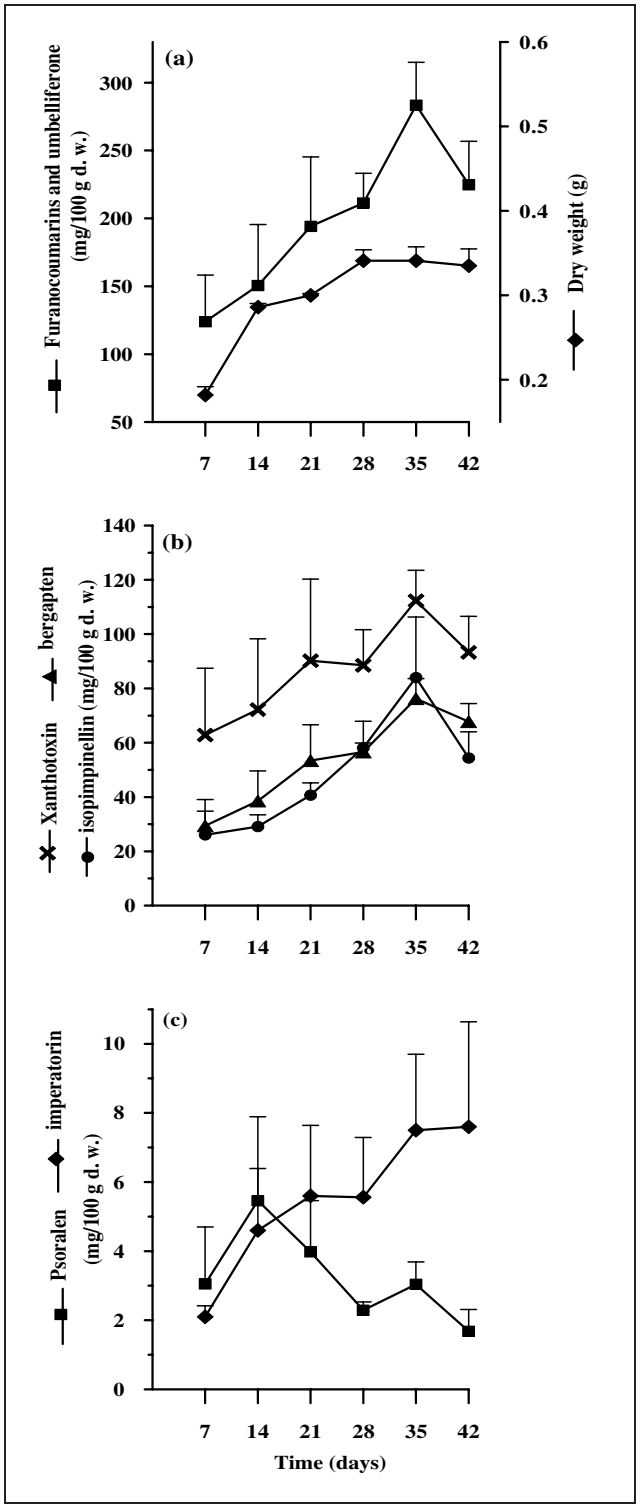

Fig.: Increase in dry biomass and total production of furanocoumarins and umbelliferone (a), and production of individual furanocoumarins (b, c) in callus tissues of *Ruta graveolens* ssp. *divaricata* (Tenore) Gams during 6-week growth cycles. The values are means ± SEM of three experiments. Since umbelliferone was detected only in few of the analysed extracts, this metabolite was not included in the figure (b), (c).

During the intensive growth phase, the total content of furanocoumarins gradually increased, which was followed by its marked, sharper rise after stationary growth phase had begun (around 28th culture day) (Fig.).

In a *Ruta graveolens* L. shoot culture maintained in the laboratory in Kraków, the end of the exponential growth phase coincided with the beginning of very intensive accumulation of the metabolites (Ekiert et al. 2001). The studies of Okazaki et al. (1982) also demonstrated that intensive accumulation of scopolin and scopoletin in *Nicotiana tabacum* L. cultures began after the termination of the exponential growth phase. The investigations of Reinhard et al. (1971) on *Ruta graveolens* L. culture showed that the onset of the exponential growth phase concurred with a proportional rise in rutamarin content. The contents of xanthotoxin, bergapten and isopimpinellin obtained in our *R. graveolens* ssp. *divaricata* culture are interesting from a practical point of view. Xanthotoxin was the main metabolite in the cultured biomass. Its maximum content of 112.3 mg/100 g d.w. was lower than in overground parts of plants growing in open air, analysed for comparison (206.7 mg/100 g d.w.). On the other hand, the maximum content of bergapten in the biomass was about 1.5 times higher than in the plant (76.2 vs. 50.2 mg/100 g d.w., respectively). Furthermore, we noted that isopimpinellin was accumulated at considerable quantities only in tissues cultured *in vitro* (max. 84.0 mg/100 g d.w.) (Table).

Table: Contents of the analysed metabolites in overground parts of *R. graveolens* ssp. *divaricata* (Tenore) Gams and their maximum amounts obtained in *in vitro* culture

Metabolites	Callus tissues		Plant material
	mg/100 g d.w.	day of growth cycle	mg/100 g d.w.
Psoralen	5.5	14	44.4
Bergapten	76.2	35	50.2
Xanthotoxin	112.3	35	206.7
Isopimpinellin	84.0	35	–*
Imperatorin	7.6	42	76.8
Umbelliferone	0.4	7	–

* Content lower than 0.001 mg/100 g d.w.

The contents of xanthotoxin obtained by us are almost 2 times higher that those reported by Massot et al. (2000) for *Ruta graveolens* L. callus tissues. These authors obtained amounts of bergapten and isopimpinellin comparable to our results. The contents of xanthotoxin and bergapten observed in our present culture are higher than quantities which we obtained earlier in *Pastinaca sativa* L. callus cultures, in which isopimpinellin dominated (Ekiert and Gomółka 2000). The biosynthetic capacity of the presently tested culture was also much higher than that of *Heracleum sphondylium* ssp. *sphondylium L.* callus culture, where Tirillini and Ricci (1998) obtained scanty contents of four furanocoumarins: bergapten, xanthotoxin, isopimpinellin and imperatorin (0.07–0.51 mg/100 g d.w.).

We propose our stationary liquid culture of *R. graveolens* ssp. *divaricata* (Tenore) Gams as an interesting material for studies of accumulation of linear furanocoumarins and as a potential source of three therapeutically important metabolites from this group, particularly xanthotoxin, and also bergapten and isopimpinellin.

3. Experimental

3.1. Establishment of in vitro culture

In vitro culture of *R. graveolens* ssp. *divaricata* (Tenore) Gams was established at the Institut für Biowissenschaften der Universität Würzburg, Germany. The starting material for the establishment of the culture originated from the plants harvested at natural sites in the Nanos Mountains in Jugoslavia in 1973, and was grown subsequently at the Botanical Garden of the above mentioned institute. Callus cultures were derived from fragments of young leaves and stems.

3.2. Experimental cultures

R. graveolens ssp. *divaricata in vitro* cultures were maintained in stationary liquid phase in Petri dishes containing glass U-tubes wrapped in filter paper, keeping biomass at the surface of the medium and allowing better infiltration of medium components. Culture conditions were described earlier (Ekiert and Gomółka 1999). The culture was maintained on Linsmaier and Skoog (1965) 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, Pila) at $25 \pm 2\,^{\circ}C$. The subcultures lasted 6 weeks. Fresh weight of biomass was determined every week. Dry mass was measured after drying of the tissue.

3.3. Plant material

Overground parts of *R. graveolens* ssp. *divaricata* harvested in July 2000 at the Botanical Garden of the Universität of Würzburg were also analysed in the present study.

3.4. Extraction and estimation of metabolites

Dried biomass from *in vitro* culture collected after 7, 14, 21, 28, 35 and 42 days was extracted according to Ekiert and Gomółka (1999). Dried callus tissue (about 1 g) was ground in a mortar and extracted with boiling 96% ethanol (two 50 ml portions, 10 h) in a Soxhlet apparatus. The extracts were combined and evaporated to dryness. The residue was quantitatively dissolved in 10 ml 96% ethanol. Extracts of plants growing in open air were prepared in identical way as extracts of the material from *in vitro* culture. The contents of furanocoumarins and umbelliferone were determined by HPLC (HPLC apparatus: Ati Unicam, Cambridge) under conditions detailed previously (Ekiert and Gomółka 1999). Separations were performed on a Supelcosil LC-8 analytical column ($4.6\,mm \times 25\,cm$) with mobile phase: methanol/water ($1 + 1.2$ v/v), and methanol/water ($2 + 1$ v/v) in case of imperatorin. The flow rate was 1.0 ml/min. Furanocoumarins and umbelliferone were detected at 310 nm. Quantification was made by comparison with reference standards manufactured by Carl Roth.

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