EFFECT OF LIGHT ON CONTENTS OF COUMARIN COMPOUNDS IN SHOOTS OF *RUTA GRAVEOLENS* L. CULTIVATED IN VITRO

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ABSTRACT

Shoots of *Ruta graveolens* L. (Rutaceae) were cultivated in stationary liquid culture under different light conditions: constant artificial light (900 lx), darkness, constant artificial light (900 lx) following irradiation with UV-C light. The contents of five furanocoumarins: psoralen, bergapten, xanthotoxin, isopimpinellin and imperatorin, as well as biogenetic precursor of these metabolites, umbelliferone, were determined by HPLC method in shoots cultivated in vitro and in overground parts of plants growing in open air. It was shown that light conditions, tested in these experiments, significantly influenced contents of the metabolites in shoots cultivated in in vitro culture. Total content of the coumarin compounds in shoots cultivated under constant artificial light (900 lx) was equal or higher than in plants growing under natural conditions. Therefore, it is suggested that stationary liquid shoot culture of *R. graveolens*. can be an alternative source for obtaining biologically active furanocoumarins.

KEY WORDS: *Ruta graveolens* L., shoots cultivated in vitro, coumarins, light conditions, HPLC analysis, plants growing in open air.

INTRODUCTION

Ruta graveolens L. (Rutaceae) is known as a rich source of, among others, numerous coumarin compounds. They include simple coumarins, coumarin dimers, linear furano- and dihydrofuranocoumarins and piranocoumarins (Hegnauer 1973; Hoppe 1973). Linear furanocoumarins, designated as psoralens, exert antiproliferative and photosensitising effects. Some of them are widely used in the treatment of many skin diseases (Pathak et al. 1981).

Our former studies led to the isolation and identification of four linear furanocoumarins: psoralen, bergapten, xanthotoxin and isopimpinellin in shoots of *R. graveolens* cultivated in stationary liquid culture (Ekiert and Kisiel 1997). The aim of the present experiments was to study the effect of light on the content of these compounds isolated from shoots cultivated in vitro. Contents of two additional metabolites, imperatorin and umbelliferone were also determined.

Influence of light on coumarin biosynthesis in tissue and cell cultures of *R. graveolens* was reported previously (Reinhard et al. 1968, 1971). However, light effect was not studied in stationary liquid shoot culture of this species.

This paper presents results of quantitative HPLC analysis of coumarin compounds in extracts of shoots cultivated in vitro and, for comparison, in overground parts of plants growing in open air.

MATERIAL AND METHODS

Culture conditions

Shoots of *Ruta graveolens* were cultivated in stationary liquid culture according to the procedure described previously (Ekiert and Kisiel 1997). Briefly, they were cultivated in Linsmaier and Skoog's (1965) medium, containing phytohormones: NAA (2 mg/dm^3) and BAP (2 mg/dm^3), at a temperature of 25 ± 2°C. The following light conditions were tested:

- constant artificial light (900 lx), LF-40 W lamp, daylight (Pila),
- constant darkness,
- constant artificial light (900 lx) following irradiation with UV-C light for 8 hours (Philips lamp).

Shoots were subcultured every 6-weeks.

Plant material

Overground parts of plants growing in open air were harvested during sunny weather in September 1997 at 3 sites in Kraków:

- Botanical Garden of the Jagiellonian University (UJ),
- Garden of Medicinal Plants of the Department of Phytochemistry, Institute of Pharmacology, Polish Academy of Sciences (PAS),
- Garden of Medicinal Plants of the Pharmaceutical Faculty, Collegium Medicum, Jagiellonian University (CMUJ).

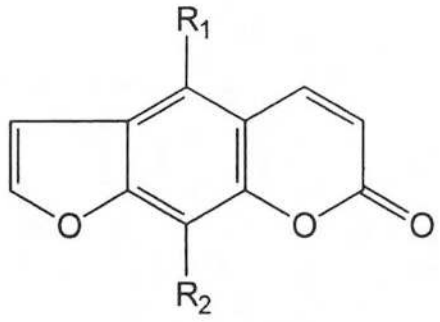
Extracts

The dried and ground shoots, cultivated in vitro under different conditions (passages from 127 to 130), and dried, ground overground parts of plants growing in open air (approximately 2 g) were extracted with 2 portions (50 cm^3) of boiling 96% ethanol in Soxhlets apparatus for 10 hours. Extracts were combined, thickened and evaporated to dryness. The residue was quantitatively dissolved in 10 cm^3 of 96% ethanol, and analysed by HPLC method.

HPLC conditions

Chromatographic quantification of psoralen, xanthotoxin, bergapten, isopimpinellin, imperatorin and umbelliferone was performed according to the procedure, developed in our laboratory. The conditions were as follows:

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 case of imperatorin: methanol – water (2: 1)
Flow rate:	1 cm^3/min.
Detector UV:	λ = 310 nm
Standards:	manufactured by Carl Roth



	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)

Fig. 1. Chemical structures of the analysed coumarin compounds.

RESULTS AND DISCUSSION

During every 6-week subculture, more than 5-fold increments of fresh weight of *Ruta graveolens* shoots were obtained, irrespective of culture light conditions. On the other hand, large differences in the increments of dry weight were noted, resulting from differences in water content in shoots growing under various conditions (5.3-fold under 900 lx, 5.9-fold – under UV-C light, 3.2-fold – in darkness).

Quantitative HPLC analysis showed significant influence of culture illumination on total content of six coumarin compounds tested: psoralen, xanthotoxin, bergapten, isopimpinellin, imperatorin and umbelliferone (Fig. 1). The maximal content of these compounds was found in shoots cultivated under constant artificial light (900 lx), lower in shoots irradiated with UV-C light and the lowest one in shoots grown in darkness (Table 1, Fig. 2).

Similar results were obtained in analogous studies on tissue cultures of *Ammi majus* L. (on agar). The highest coumarin contents were observed when the cultures were maintained under constant artificial light (900 lx) and they were significantly lower (3 times on the average) under other light conditions (darkness, UV-C light) (Ekiert and Gomółka 1997). Results of Reinhard et al. (1971) are also in line with this findings as they reported much lower total content of the discussed coumarins in tissue cultures of *R. graveolens* conducted in darkness in comparison with those maintained under constant light (60-80 lx).

Light influenced also the content of individual coumarins in shoots cultured in vitro. The highest content of one metabo-

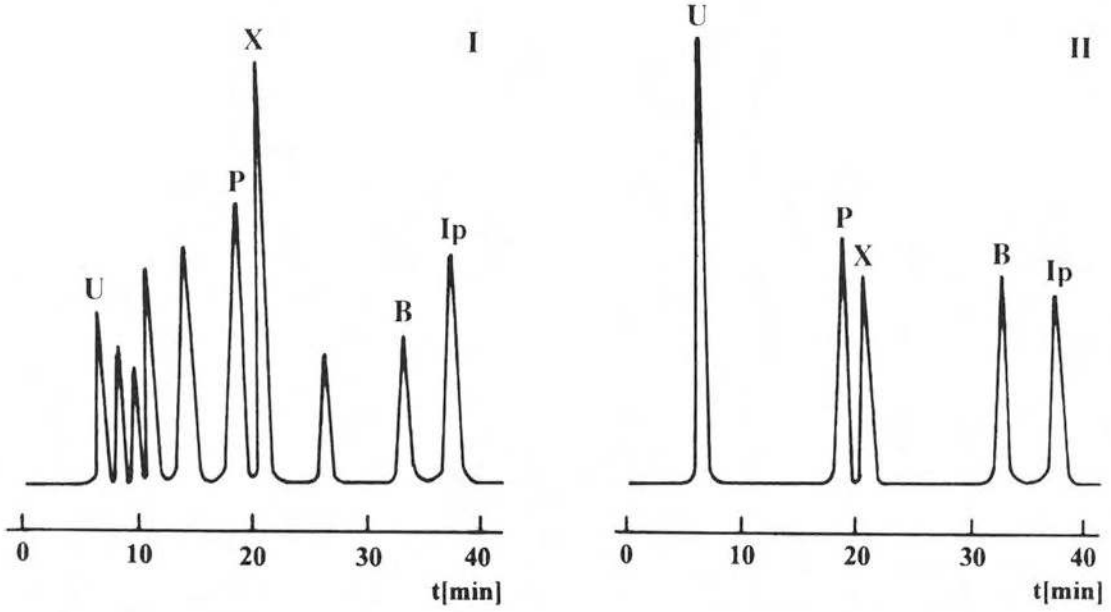


Fig. 2. Separation of coumarin compounds. I – extract from shoots of *Ruta graveolens* L. cultivated under continuous light (900 lx), II – mixture of coumarin standards; U – umbelliferone, P – psoralen, X – xanthotoxin, B – bergapten, Ip – isopimpinellin.

TABLE 1. Content of coumarin compounds (mg%)* in overground parts of *Ruta graveolens* growing in open air and their maximal amounts in shoots cultivated in vitro.

Metabolite	Plants growing in open air			Shoots cultivated in vitro **		
	Garden of CMUJ	Botanical Garden of UJ	Garden of PAS	900 lx	darkness	UV-C
Psoralen	132.9	218.2	125.2	143.7	30.2	125.0
Xanthotoxin	230.4	444.1	237.8	433.4	66.3	350.5
Bergapten	0.0	39.4	26.1	198.4	162.6	177.3
Isopimpinellin	174.3	282.1	215.3	219.5	159.1	181.8
Imperatorin	2.3	1.8	0.0	5.9	5.5	5.4
Umbelliferone	34.8	47.5	28.8	21.3	13.3	11.6
Total	574.7	1033.1	633.2	1022.2	437.0	851.6

* mg/100 g of dry weight; ** mean values of 4 subcultures

lite, equalling 433.4 mg% was observed in case of xanthotoxin (900 lx). Maximal contents of isopimpinellin (900 lx) and bergapten (900 lx) were also high, approximating 200 mg%. These results are of special interest since just these metabolites, xanthotoxin, bergapten and isopimpinellin, are pharmacologically active substances, and the most frequent components of antiproliferative and photosensitising preparations (Wolff and Tessa 1986).

Studies of Reinhard et al. (1968) yielded similar results as they indicated that xanthotoxin was a dominating metabolite in callus tissue culture of *R. graveolens*. Other investigations of Reinhard et al. (1971) showed 3(1',1'-dimethylallyl)7,8-dimethoxycoumarin to be the main metabolite, and among the compounds, discussed in this work, bergapten was detected at the highest concentrations. Contents of isopimpinellin were also high, but quantities of xanthotoxin and psoralen were lower.

Imperatorin contents were definitely low, approximating several mg%, in shoots from our in vitro culture. It explains our difficulties to isolate this metabolite. We plan to isolate this compound and confirm its chemical structure by spectral methods, the more so as no literature data have been found on its presence in cultures of *R. graveolens* (Petit-Paly et al. 1986) while its isomer, isoimperatorin, was detected in overground parts of this species growing under natural conditions (Reisch et al. 1966).

We detected also only minor levels of umbelliferone, precursor of all furanocoumarins, in shoots cultivated in in vitro cultures. This result and high furanocoumarin content, observed in our experiments, indicate possible incorporation of this compound into more complex structures. In Reinhard's et al. (1968) studies, umbelliferone content was also low in callus culture of *R. graveolens*.

In shoots irradiated with UV-C light, contents of all tested compounds were proportionally lower in comparison with shoots cultivated under visible artificial light. In darkness, further significant decrease in contents of all compounds under examination was noted, which was most pronounced in case of xanthotoxin and psoralen, and less apparent, but noticeable for isopimpinellin and bergapten (Table 1). Similar relationship was observed in analogous experiments on tissue cultures of *Ammi majus* L., mentioned above, which indicated considerable, 3-fold on the average, drop not only in xanthotoxin level but also in that of isopimpinellin and bergapten (Ekiert and Gomółka 1997). An opposite effect was observed in tissue cultures of *Ammi majus* L. of other origin, i.e. a significant increase in bergapten content in darkness (Ekiert 1993). On the other hand, Reinhard et al. (1971) reported different influence of light on coumarin biosynthesis in two distinct *Ruta graveolens* L. cell lines, however they tested disparate light conditions: light with an intensity of 60-80 lx and 2500 lx or darkness.

In tissue cultures of *Pastinaca sativa* L. maintained in darkness, a significant decrease in isopimpinellin content was observed while concentrations of other metabolites remained at the same level as in case of lit cultures (900 lx) (Ekiert, unpublished data).

In overground parts of plants growing in open air, also xanthotoxin, isopimpinellin and psoralen dominated quantitatively while imperatorin and umbelliferone were detected only at minor quantities. Contrary to the material obtained in vitro, just minute levels of bergapten occurred in specimens from in vivo cultures.

Comparison of total contents of all tested compounds in material growing in vivo and cultured in vitro indicates that in shoots cultivated in vitro under constant light with an intensity of 900 lx, quantities of coumarins are higher or equal to those observed in overground parts of plants growing in open air. Examples of such ample efficiency of the biosynthesis of secondary metabolites in vitro are rare (Chmiel 1992). Therefore, stationary liquid shoot culture of *R. graveolens* maintained under constant light (900 lx) can be considered an alternative source of biologically active coumarins, the source which is independent of seasons, weather conditions, plant diseases, etc. It can be proposed that this culture can be a suitable biological model in the studies on optimisation of biosynthesis of coumarins.

It should be emphasised that high content of active substances in shoots cultivated under constant light is accompanied by good increments of both fresh and dry weight, which can be of practical importance.

Large differences in content of coumarins in overground parts of plants growing in open air can be explained by the influence of a variety of environmental factors on their accumulation. Such diversity in the material from in vivo culture is a further argument substantiating the possibility to control conditions of biosynthesis in vitro. Another characteristic of our experimental model, which is worthy of stressing, is age of our shoot culture (15 years) and its constant biosynthetic potential, which has already been brought into attention earlier, during the analysis of material 7.5, 10 and 12.5 years old (Ekiert 1994).

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LITERATURE CITED

CHMIEL A. 1992. Biotechnologia komórek roślinnych. Biotechnologia 4: 5-16.

EKIERT H. 1993. *Ammi majus* L. (Bishops weed): In vitro culture and the production of coumarin compounds. In: Biotechnology in Agriculture and Forestry. Vol. 21. Medicinal and Aromatic Plants IV., Bajaj Y.P.S. (ed.), Springer Verlag, Berlin-Heidelberg, 1-17.

EKIERT H. 1994. Metabolites of *Ruta graveolens* L. shoot culture. W: Potencjalne zastosowania kultur in vitro w nowoczesnej hodowli roślin. Mat. VII Ogólnopol. Konf. Kultur in Vitro. Ustroń: s. 65.

EKIERT H., GOMÓŁKA E. 1997. Wpływ warunków hodowli in vitro na zawartość kumaryn w tkance kalusowej *Ammi majus* L. i pędach *Ruta graveolens* L. W: Lek roślinny – tradycja i współczesność. Mat. Konf. Nauk., Kraków: s. P-19.

EKIERT H., KISIEL W. 1997. Coumarins and alkaloids in shoot culture of *Ruta graveolens* L. Acta Soc. Bot. Pol., 66: 329-332.

HEGNAUER R. 1973. Chemotaxonomie der Pflanzen. Birkhäuser Verlag, Basel, Bd. 6, 174-230.

HOPPE A. 1973. Drogen Kunde. Walter de Gruyter Verlag, Berlin-New York, Bd.2, 949-952.

LINSMAIER E.M., SKOOG F. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18: 100-127.

PATHAK M.A., PARRISH J.A., FITZPATRICK T.B. 1981. Psoralens in photochemotherapy of skin diseases. Farmaco, Ed. Sci. 36: 479-491.

PETIT-PALY G., RAMAWAT K.G., CHENIEUX J.C., RIDEAU M. 1986. *Ruta graveolens*: In Vitro Production of Alkaloids and Medicinal Compounds. In: Biotechnology in Agriculture and Forestry. Vol. 7. Medicinal and Aromatic Plants II., Bajaj Y.P.S. (ed.) Springer Verlag, Berlin-Heidelberg, 488-505.

REINHARD E., CORDUAN G., VOLK O.H. 1968. Über Gewebekulturen von *Ruta graveolens*. Planta Med. 16: 8-16.

REINHARD E., CORDUAN G., BROCKE W. 1971. Untersuchungen über das ätherische Öl und die Cumarine in Gewebekulturen von *Ruta graveolens*. Herba Hung. 10: 9-26.

REISCH J., NOVAK I., SZENDREI K., MINKER E. 1966. Isoimperatorin als Inhaltsstoff von *Ruta graveolens* L. Pharmazie 29: 628-629.

WOLFF F.A., TESSA T.V. 1986. Clinical pharmacokinetics of methoxsalen and other psoralens. Clin. Pharmacokin. 11: 62-75.

WPŁYW ŚWIATŁA NA ZAWARTOŚĆ POŁĄCZEŃ KUMARYNOWYCH W PĘDACH *RUTA GRAVEOLENS* L. HODOWANYCH IN VITRO

STRESZCZENIE

Płynną stacjonarną hodowlę pędów *Ruta graveolens* L. (Rutaceae) prowadzono w różnych warunkach świetlnych: ciągłe sztuczne oświetlenie – 900 lx, ciemność, ciągłe sztuczne oświetlenie – 900 lx po uprzednim naświetleniu promieniowaniem UV-C. W pędach z hodowli in vitro oraz w częściach nadziemnych roślin rosnących w warunkach naturalnych, techniką HPLC ilościowo oznaczono pięć furanokumaryn: psoralen, bergapten, ksantotoksynę, izopimpinelinę i imperatorynę oraz biogenetycznego prekursora tych metabolitów – umbeliferon. Wykazano wyraźny wpływ testowanych warunków świetlnych hodowli in vitro na zawartość poszczególnych metabolitów. W pędach z hodowli prowadzonych przy ciągłym sztucznym oświetleniu (900 lx) całkowita zawartość oznaczanych związków była równa lub wyższa niż w materiale ze stanu naturalnego. Płynną stacjonarną hodowlę pędów *R. graveolens* zaproponowano jako potencjalne alternatywne źródło pozyskiwania biologicznie aktywnych furanokumaryn.

SŁOWA KLUCZOWE: *Ruta graveolens* L., pędy hodowane in vitro, kumaryny, warunki świetlne, analiza HPLC, rośliny rosnące w warunkach naturalnych.