Characterization of Volatile Constituents from Heterotrophic Cell Suspension Cultures of *Ruta graveolens*

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Heterotrophic cell suspension cultures of *Ruta graveolens* were established by reversion of photomixotrophic cultures without any change in the chemical composition of the growth medium. The heterotrophic cultures were qualitatively and quantitatively analyzed by gaschromatography and mass spectroscopy for volatile compounds. The terpenoid hydrocarbons geijerene and pregeijerene, C_6-C_8 ketones, acetic acid *n*-butylester and a series of aliphatic C_4-C_9 primary and secondary alcohols were found as main constituents. Two isomeric sabinene hydrates were also isolated as new constituents of rue cells. The data are discussed in comparison to results obtained with photomixotrophic cell suspension cultures.

Introduction

Comparative studies on secondary constitutents in photosynthetically active and heterotrophic cell cultures of higher plants have shown that the mode of carbon supply and the pattern of cellular differentiation greatly determines the spectrum of accumulating compounds [1–6]. Reversion of photoautotrophic or photomixotrophic cell suspension cultures to heterotrophic cells and *vice versa* represents a means to determine whether the presence or absence of functioning chloroplasts contributes to secondary product formation [4, 5, 7].

In continuation of our analyses of volatile constituents from photomixotrophic cell suspension cultures of *Ruta graveolens* [8] we have now reversed these cells to heterotrophic cell cultures and here report a detailed investigation of the volatiles from non-green rue cell cultures. These studies again prove the different potential for secondary metabolism of heterotrophic and photosynthetically active cell cultures because the high yields of aliphatic esters characteristic for the photomixotrophic cultures have not been found in heterotrophic cells.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, mass spectroscopy; GC, gaschromatography; RT, retention time.

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Materials and Methods

Cell cultures

Photomixotrophic cell suspension cultures as grown for previous investigations [8] were reversed to heterotrophic cell cultures by incubation in complete darkness without any alteration in the nutrient medium. The suspensions were kept in 200 ml Erlenmeyer flasks with 40 ml medium and 2,4-D as phytohormone. All other details of the culture procedure and the collection of cells were essentially as previously described [8]. The reversion process was followed by measuring the decrease of the chlorophyll content [8].

Extraction procedure

Our established methods for the extraction of frozen (−40 °C) cells (250 g) and for the fractionation of cellular constituents were as described earlier [8–10].

Gaschromatography and mass spectroscopy

The procedures for gaschromatography of volatile constituents and their identification by mass spectroscopy have been reported [8, 11]. The quantita-

tive determination of all compounds using undecanecarboxylic acid methylester as reference compound has also been outlined in earlier reports [8, 11].

Reference compounds

(*E*)- and (*Z*)-sabinene hydrate were synthesized from sabinene according to [16]. All other reference compounds were from the collection of the Weihenstephan institute.

Results

Without change of the culture medium the photomixotrophic *Ruta graveolens* cell suspension cultures as used in previous experiments [8] were turned into heterotrophic [4] cell suspensions by incubation in darkness. The growth yield was hereby somewhat reduced though these suspensions finally yielded 5 ± 0.2 g fresh weight from 1 g inoculum in 14 days. After 4–5 passages the chlorophyll content had dropped below the level of detection. Cells for determination of volatiles were harvested in early stationary phase.

Isolation and structural elucidation of volatile compounds

The total methanol extract [9] of 250 g heterotrophic rue cells was fractionated into 3 fractions and analyzed by gaschromatography-mass-spectroscopy [8]. The identification of compounds and their quantitative determination were carried out essentially as in our earlier experiments [8–11].

Fraction 1 (Table I) contained a series of hydrocarbons which could only partially be identified. Geijerene and pregeijerene were again found as the most prominent constituents in this fraction. The complete structural elucidation of the various isogeijerene compounds warrants further investigations though these products appear to be artifacts formed during the isolation procedure.

Fraction 2 (Table II) mostly contained a series of ketones and, as the main constituent, acetic acid *n*-butylester in addition to small amounts of various esters of aliphatic and aromatic acids. In contrast to the photomixotrophic rue cultures [8] aliphatic aldehydes and primary alcohols could not be detected in this fraction.

In fraction 3 (Table III) low amounts of numerous primary and secondary alcohols could be identified which have also not been found in free form in the green cell cultures of *R. graveolens*. Quite surprisingly the two isomeric sabinene hydrates were found as hitherto unreported constituents of rue cells or plants. Other terpenoids previously reported to occur in *R. graveolens* such as myrcene and linalool as well as limonene, 1,8-cineol and pinene [12] were absent from the cell extracts. According to Maarse *et al.* [13] such compounds might be artifacts originating from improper isolation procedures such a prolonged steam distillation of volatiles.

The quantitative data in Tables I–III on the c currence of volatile constituents in *R. graveolens* heterotrophic cell cultures document that most of the compounds occur in very small amounts only. Except for pregeijerene all compounds occur in much lower

Table I. GC–MS analysis and quantity of hydrocarbons (fraction 1) obtained from heterotrophic cell suspension cultures of *Ruta graveolens*. The retention index (R_I) of each compound was determined on a carbowax 20-M column using a defined temperature gradient [9]. The mass fragments *(m/e)* are arranged in decreasing intensity with M^+ indicating the largest mass fragment observed.

R_I	Compound	Mass fragments *(m/e)*	M^+	Quantity (µg/kg fr.w.)
1315	Geijerene derivative	79, 94, 41, 91, 77	147	n.m.
1325	Geijerene	79, 94, 41, 77, 91	162	480
1429	Isogeijerene A	91, 105, 41, 92, 77	162	n.m.
1473	Isogeijerene B	91, 79, 147, 105, 41	162	n.m.
1492	Isogeijerene C	91, 147, 41, 105, 79	162	n.m.
1536	Isogeijerene D	147, 91, 41, 105, 162	162	n.m.
1577	Pregeijerene	79, 94, 41, 77, 91	162	1040

n.m. = not measured.

Table II. GC–MS analysis and quantity of ketones and esters (fraction 2) obtained from heterotrophic cell suspensions of *Ruta graveolens*. Means of identification were comparison of mass spectra (MS) and of GC-retention times (RT) with authentic reference compounds.

Compound	Identification	Quantity (μg/kg fr.w.)
3-hexanone	MS, RT	80
2-hexanone	MS, RT	200
3-heptanone	MS, RT	240
2-heptanone	MS, RT	60
4-octanone	MS	10
3-octanone	MS, RT	80
2-octanone	MS, RT	20
2-nonanone	MS, RT	15
2-decanone	MS, RT	<10
2-undecanone	MS, RT	80
2-tridecanone	MS, RT	20
iso-octanone ($C_8H_{16}O$)	MS	<10
5-nonanone	MS, RT	20
p-menthan-4-ol	MS, RT	25
acetic acid-*n*-butylester	MS, RT	1000
-*n*-nonylester	MS, RT	20
-2-undecylester	MS, RT	50
2-methylbutyric acid-octyl ester	MS, RT	<10
3-methylbutyric acid-octyl ester	MS, RT	<10
benzoic acid methyl ester	MS, RT	25
phenylacetic acid methyl ester	MS, RT	40

Table III. GC–MS analysis and quantity of aldehydes and alcohols (fraction 3) obtained from heterotrophic cell suspensions of *Ruta graveolens*. Means of identification as mentioned in legend of Table II.

Compound	Identification	Quantity (μg/kg fr.w.)
(*E*)-sabinene hydrate	MS, RT	25
(*Z*)-sabinene hydrate	MS, RT	70
malonic acid dimethylester	MS, RT	<10
3-pentene-2-one	MS, RT	20
2-methyl-2-butenal	MS	60
(*E,E*)-2,4-hexadienal	MS, RT	25
decanal	MS, RT	10
benzaldehyde	MS, RT	<10
2-pentanone	MS, RT	20
2-methylbutan-2-ol	MS	20
butan-2-ol	MS, RT	100
pentan-2-ol	MS, RT	20
heptan-3-ol	MS, RT	20
octan-4-ol	MS, RT	<10
octan-3-ol	MS, RT	<10
nonan-5-ol	MS, RT	<10
2-methylpropanol	MS, RT	200
n-butanol	MS, RT	100
n-pentanol	MS, RT	40
n-hexanol	MS, RT	20
n-octanol	MS, RT	10

quantity than measured for the volatiles of green rue cells [8].

Discussion

These and our previous studies [8] based on detailed and highly sensitive analyses document that cell suspension cultures of *R. graveolens* possess the potential to form a large number of volatiles. In view of the comparatively low yield of the constituents it must be remembered that no selection processes for high yielding cell lines have been conducted so far.

The structures of the compounds listed in Tables I to III also show that a variety of different secondary biosynthetic pathways must be expressed in these heterotrophic rue cells. Formation of the hydrocarbons geijerene and pregeijerene which are root constituents of *R. graveolens* [15] is again found (Table I). Expression of this biosynthetic pathway in cell cultures appears to be largely independend from the type of cell culture because the photomixotrophic rue cell suspensions [8] showed the same property though in much smaller amounts.

In general agreement with previous researchers [1, 2, 14] using green and heterotrophic callus cultures the chemical composition of the volatiles of light-grown and dark-grown *R. graveolens* cells is strikingly different. Our detailed GC–MS data clearly prove these differences (Tables II, III and [8]).

Thus, the characteristic constituents of photomixotrophic cells are the C_9-C_{13} methylketones and a series of esters of 2-methylbutyric acid and 3-methylbutyric acid esterified with straight chain or branched C_8-C_{11} alcohols. Except for trace amounts of 2-undecanone, 2-tridecanone and the octylester of 2-methylbutyric acid and 3-methylbutyric acid these compounds are practically absent from the heterotrophic cells. It is safe to postulate that these characteristic constituents of photosynthetically active cells are the result of chloroplast metabolism.

On the other hand, heterotrophic rue cells produce a complex mixture of C_6-C_8 ketones, acetic acid *n*-butylester and a series of C_4-C_9 primary and secondary alcohols which have not been found in the green cell cultures. It must again be mentioned that this pronounced shift in the pattern of secondary volatile constituents is the result of reversion of a green to a non-green culture without any change in the chemical composition of the nutrient medium and without any exogenous alteration of the phytohormonal situation of the cells.

Further application of reversion experiments of green to non-green cells and vice versa obviously offers a means to analyze how secondary biosynthesis is effected by cellular carbon metabolism and by the presence of functioning chloroplasts. Furthermore, comparative studies using these two types of plant cell cultures obviously widens the applicability of the cell culture technique for investigations on leaf and/or root constituents of higher plants because the establishment of better producing systems seems to be more feasable.

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