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LIPIDS OF THE BIOMASS OF *Ruta graveolens*, GROWN *in vivo* AND *in vitro*

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A lower content of chlorophylls, phospholipids, and linolenic acid and a higher content of rutin, carotenoids, and xanthophylls in the biomass of Ruta graveolens *L. obtained in vitro has been found as compared with these indices from the epigeal part of the plant grown* in vivo.

The biomass of medicinal plants obtained *in vitro* by callusogenesis and regeneration may be a valuable raw material for preparation of galenical preparations and the extraction of biologically active substances such as alkaloids, vitamins, and organic acids [1, 2]. Information on the production of bioactive compounds of lipid nature by the *in vitro* cultivation of medicinal plants is sparse. It has been found that, in comparison with the intact plants, callus cultures are characterized by relatively high contents of steroids and steryl glycolipids, in view of which it is desirable to use this method for their production [2].

Ruta graveolens L. (common rue, family Rutaceae) is a perennial essential-oil and medicinal plant [3]. In the wild form, it is widespread in the Mediterranean from the Canary Islands to Asia Minor and the Near East, and under natural conditions it grows in Southern Crimea, while it is cultivated in the Ukraine, in Moldova, and in the Baltic region [4, 5]. *R. graveolens* is finding wide use in folk medicine and scientific medicine in the treatment of gastric, nervous, gynecological, skin, kidney, and other diseases [5].

With the aim of broadening the variety of medicinal plants in Uzbekistan, the Institute of Botany of the Academy of Sciences of the Republic of Uzbekistan is conducting investigations on the multiplication of *R. graveolens* in the open and, in parallel, is studying the possibility of obtaining a biomass (raw material) by the method of cultivating tissues *in vitro*. There is information in the literature on coumarins, alkaloids, essential oil, carotenoids, and chlorophylls of the intact plant and of a callus tissue of *R. graveolens* [1], but the lipids have not been characterized. The material used for investigating the lipids consisted of one-year leafy shoots in the period of mass flowering and at the beginning of fruit-bearing from *R. graveolens* growing in the open (I) and from 45-day plants obtained in a flask. The *in vitro* culture was grown in a known medium using phytohormones. Of the phytohormones that we tested — indol-3-ylacetic acid, kinetin, and 2,4-dichlorophenoxyacetic acid —, the most active for stimulating organogenesis proved to kinetin in low concentrations.

The investigation showed that the cells of an explant of *R. graveolens* in a nutrient medium entered into active division fairly rapidly, as a consequence of which shapeless calluses of dense consistency were formed. These possessed a well-defined morphogenic capacity which is probably characteristic for this genotype. The conditions of *in vitro* cultivation were regulated with the aim of intensifying the growth and accumulation of a green biomass.

The biomass of the plants *in vitro* was separated into differentiated tissue (shoots and leaves, II) and callus tissue (III). The moisture content and amount of rutin in the fresh biomasses (I) and (II) were determined. The pigments were isolated from part of the biomasses (I) and (II) with acetone. The lipids were extracted from biomasses (I) and (II) with a mixture of chloroform and isopropanol after the tissues had first been fixed with hot isopropanol. Lipids (I) and (II) were fractionated by countercurrent distribution and by CC. The fatty acids (FAs) were isolated from the total lipids (I) and (II) and from the glycolipids (GLs) and phospholipids (PLs) of (I) and (II), and, in the form of methyl esters, were analyzed by GLC.

The moisture content of biomass (I) was 76% and that of (II) 87%; the amounts of lipids, pigments, and rutin that they contained are given in Table 1 and the fatty acid compositions of their lipids in Table 2.

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TABLE 1. Amounts of Lipids, Pigments, and Rutin in the Biomasses (I) and (II) from *R. graveolens*

Component	I		II	
	mg/g	mg/g a.d.m.	mg/g	mg/g a.d.m.
Lipids, and of these:	20.4	87.0	13.2-15.7	98-117
neutral	9.4	40.0	7.4-8.8	55.5-66.2
glycolipids	6.0	25.6	3.4-4.0	25.1-30.1
phospholipids	5.0	21.4	2.3-2.7	17.3-20.7
chlorophyll a	0.92	4.0	0.47	3.54
chlorophyll b	0.60	2.6	0.33	2.45
Carotenoids and xanthophylls	0.21	0.90	0.18	1.34
Rutin	0.71	3.1	0.72	4.6

TABLE 2. Fatty-Acid Compositions of the Lipids of Biomasses (I)-(III) of *R. graveolens* (%, GLC)

Acid	Total lipids			Glycolipids		Phospholipids	
	I	II	III	I	II	I	II
12:0	Tr.	Tr.	Tr.	0.2	0.2	0.1	0.1
14:0	2.5	1.6	1.5	0.4	0.5	0.2	1.5
15:0	1.6	0.6	1.2	0.3	Tr.	Tr.	0.6
15:1	0.6	0.5	Tr.	Tr.	Tr.	Tr.	Tr.
16:0	14.7	18.0	18.3	5.4	12.0	22.8	26.3
16:1	0.8	0.7	0.5	0.9	0.9	4.1	0.7
17:0	2.6	0.7	2.1	1.1	1.1	Tr.	Tr.
17:1	2.0	1.3	1.7	Tr.	Tr.	Tr.	Tr.
18:0	1.9	2.2	2.6	3.5	1.8	2.1	4.1
18:1	5.7	4.9	5.7	3.6	2.2	2.4	5.5
18:2	15.5	26.9	34.9	7.8	26.7	38.8	43.1
18:3	52.1	42.6	31.5	76.8	54.6	29.5	18.1
Σ_{sat}	23.3	23.1	25.7	10.9	15.6	25.2	32.6
Σ_{unsat}	76.7	76.9	74.3	89.1	84.4	74.8	67.4

The yields of lipids from the biomass of the *in vitro* culture in different batches of the sample varied, which is connected with an instability of the conditions of growth and amounted on average 10.7% a.d.m. as compared with 8.7% a.d.m. from the biomass of an intact plant. With respect to the level of lipids per 1 g of crude tissue, biomass (II) was 1.3-1.5 times poorer in view of its higher moisture content. On an average, there was 1.5 times more neutral lipids in biomass (II) than in (I), while the fluctuations in the amounts of GLs and PLs were insignificant. If we compare the amounts of individual groups of lipids in their total amounts in biomasses (I) and (II), they amounted to 45.9 and 56.6% for the NLs, 29.4 and 25.7% for the GLs, and 24.7 and 17.7% for the PLs, respectively. These figures show that the lipids of the photosynthetic tissues of *R. graveolens* in the given phase of development were characterized by a predominance of neutral lipids, and, regardless of the batches of material, this predominance was more pronounced in tissues (II). In the other groups of lipids of (II), with a slight fall in the proportion of GLs the proportion of PLs was 1.4 times less than in the case of (I).

The amount of lipids in tissues (III) was very small (14.0 mg/g a.d.m.) and they were therefore not separated into individual groups of lipids. The qualitative compositions of the three groups of lipids of biomasses (I) and (II), studied by TLC in comparison with standards and with specific revealing agents, proved to be identical. In the neutral lipids, besides carotenoid and chlorophyll pigments, we detected saturated and unsaturated hydrocarbons, esters of FAs with alkanols and cyclic alcohols, triacylglycerols, free FAs, phytosterols, and a number of unidentified compounds, including pigments (yellow, orange, and pink).

The glycolipids consisted of mono- and digalactosyldiacylglycerols, steryl glycosides and their esters, and sulfoquinovosyldiacylglycerols. In the PLs we identified phosphatidylethanolamines, phosphatidylcholines, phosphatidylinositols, phosphatidylglycerols, and phosphatidic acids.

It can be seen from Table 2 that the total lipids of biomasses (I) and (II), while having the same sets of FAs with similar degrees of unsaturation, differed substantially with respect to their contents of the 18:2 and 18:3 acids. In lipids (I), more than 50% of the total consisted of the 18:3 acid, which is characteristic for actively functioning chloroplasts [6]. In the lipids of the callus material, having no differentiated photosynthetic tissues, there was somewhat more of the 18:2 than of the 18:3 acid. The FAs of biomass (II) differentiated from callus, contained less of the 18:2 acid but more of the 18:3 acid than in the callus itself. In comparison with the tissues of the intact plant, in both lipids of biomass (II), the level of the 18:3 and 16:0 acids was 1.2 times smaller and, simultaneously, the level of the 18:2 acid was 1.7 times greater.

Substantial differences were observed in the fatty acid compositions of the GLs and PLs participating in the structural-functional organization of the membranes, including the photosynthetic membranes [7].

It can be seen from a spectrophotometric analysis of the pigments of the sample studied (Table 1), that the amounts of total pigments in mg/g a.d.m. in the biomasses (I) and (II) had similar values (7.5 and 7.33 mg/g) but in the *in vitro* culture there was some decrease in the amount of chlorophylls and an increase in the amount of carotenoids and xanthophylls. The UV spectrum of acetone extracts showed an absorption band of β-carotene at λ_{max} 427, 454, and 480 nm with weak inflections at 424, 448 and 476 nm which corresponded to β-carotene [8]. The presence of α-carotene (R_f 0.19) with a small amount of the α- isomer (R_f 0.27) [sic] was confirmed by TLC on Al_2O_3.

The amount of rutin in mg/g a.d.m. in the biomass (II) was almost 1.5 times higher than in (I).

Thus, in the biomass of a 45-day *in vitro* culture of *R. graveolens* a fall in the concentrations of chlorophylls, PLs, and the 18:3 acid and a rise in the proportion of a xanthophylls, neutral lipids and the 18:2 acid in the GLs and PLs as compared with these indices for the epigeal part of an *in vivo* culture were observed. If it is borne in mind that chlorophyll pigments, membrane lipids, and the 18:3 acid are characteristic for a functionally active photosynthetic apparatus [6], the decrease in their amount apparently reflects a fall in the capacity of the *in vitro* culture for performing full-value photosynthesis. However, in spite of the lower photosynthetic activity of biomass (II), its capacity for biosynthesizing neutral lipids and rutin was not only no lower than that of biomass (II) but was actually higher. The biomass of the *in vitro* culture of *R. graveolens* is capable of synthesizing the main components of the *in vivo* culture and in future may serve as a source of the biologically active lipids, rutin, and pigments, that are characteristic of the intact plant.

EXPERIMENTAL

The UV spectra of the pigments were recorded on a Hitachi spectrophotometer in acetone. The optical densities of solutions of rutin were measured in a KFK-2 concentration photoelectric colorimeter, type MI 1236-86, at λ 364 nm.

The GLC of the FA methyl esters was conducted on a Chrom-4 chromatograph (Czechoslovakia) with a flame-ionization detector using a 3 mm $\times$ 3.5 mm column filled with Chromaton NAW-DMCS impregnated with 15% of Reoplex 400 at 196°C, the carrier gas being helium.

The CC and countercurrent distribution of the lipids have been described in [9].

The TLC of the lipids was conducted on silica gel L 5/40 that had been washed with a mixture of chloroform and methanol (2:1) and activated at 110°C with 10% of $CaSO_4$. The solvent systems used for TLC were hexane–ether (95:5), hexane–ether–CH_3COOH (7:30:1) (neutral lipids); hexane–acetone–benzene–isopropanol (69.5:25:4:1.5) (pigments); and chloroform–acetone–methanol–CH_3COOH–H_2O (65:20:10:10:3 (GLs). The two-dimensional GLC of the PLs was conducted in the systems chloroform–methanol–NH_4OH (14:6:1) (direction I) and chloroform–methanol CH_3COOH–H_2O (20:6:1:1) (direction II). TLC was conducted on Woelm brand acidic Al_2O_3 sorbent (Germany) in hexane. The substances were revealed on the chromatograms with I_2 vapor and with 50% H_2SO_4 followed by heating at 200°C. The following specific revealing agents were used: the Vas'kovskii reagent for PLs, the Dragendorff reagent for phosphatidylcholines, the ninhydrin reagaent for phosphatidylethanolamines, and α-naphthol for GLs. The classes of lipids were identified as described in [9].

We investigated *R. graveolens* plants grown in an open plot in the Tashkent Experimental section of the Institute of Botany of the Academy of Sciences of the Republic of Uzbekistan. For obtaining and growing the *in vitro* culture we used the methodological expedients proposed in handbooks [10] and the Murashige-Skoog nutrient medium with slight modifications

to its composition. The explants sown in the sterile nutrient medium (in 250-ml flasks) were cultivated in a factorostatic chamber at 26 ± 2°C at humidity of 70% with illumination of 3-4 thousand lux.

The extraction of the lipids from the biomass of the plants with inactivation of enzymes, and the isolation of the FAs and the preparation of the methyl esters have been described in [9].

The amounts of pigments were determined spectrophotometrically [11] and that of rutin as described in [12].

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