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SYNTHESIS OF VOLATILE OILS IN TISSUE CULTURES OF *RUTA GRAVEOLENS**

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Abstract—Callus cultures of *Ruta graveolens* were used to investigate the influence of several factors, independently, on volatile oil composition, in an attempt to explain observed differences in oil composition between the organs of this species. Autotrophic or heterotrophic conditions did not affect composition. However, drastic differences were found between light- and dark-grown callus cultures. The composition of light-grown cultures corresponded to that of leaf tissue. The effect of light does not depend on photosynthesis. Light-grown cultures placed in darkness altered their composition until, after 11 weeks, the volatile oils were similar to those in the roots of the plant.

INTRODUCTION

CALLUS CULTURES of *Ruta graveolens* are so far the only tissue cultures capable of synthesizing volatile oil. This volatile oil is excreted in schizogenous passages that are located mainly near the surface of the tissue pieces or are distributed within the tissue. The anatomical features of the secretory passages in the tissue cultures resemble those of the differentiated plant. The volatile oil may also be excreted in parenchyma cells in the form of small droplets.[1] Comparison of the components of the volatile oil of the organs of the differentiated plant[2] of *Ruta graveolens* with those of the tissue cultures[1] showed that the leaf oil is most similar to the volatile oil of tissue cultures grown in the light. In both cases the main components are undecanone, nonanone and their acetates. We also found that the composition of the oil of cultures kept in the dark is different from that of cultures grown in light.

The different composition of the volatile oil in the organs of the differentiated plant can be caused by different conditions.[3] In the autotrophic parts of the plant (leaves, green stems and fruits) there are apparently mainly secretory passages, in contrast to heterotrophic roots and stems covered by a bark, which form undifferentiated oil cells. That the different oil composition does not depend on the site of secretion, oil cell or secretory passages, has already been shown by experiments with tissue cultures. We also demonstrated that different nutrient media did not influence the composition of the volatile oil.[4]

In the present study further experiments were carried out to investigate to what degree the secretion of volatile oil and its composition depend on factors such as the intensity of light,

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[1] E. REINHARD, G. CORDUAN and O. H. VOLK, *Planta Med.* **16**, 8 (1968).
[2] K. K. KUBECZKA, *Flora Abs.* A. **158**, 519 (1967).
[3] E. SPRECHER, *Planta Med.* Suppl. 87 (1967).
[4] G. CORDUAN, O. H. VOLK and E. REINHARD, *Dtsch. Ap. Ztg.* **107**, 1411 (1967).

the age of tissue, or culture under autotrophic or heterotrophic conditions. In this way it was hoped to reach conclusions on the condition which determine the different oil compositions in the organs of the differentiated plant.

RESULTS

The Influence of Different Light Intensity on Growth and Oil Composition

In an earlier study[1] we demonstrated that the oil composition of cultures maintained in light differs from those maintained in dark. To determine the light intensity necessary to obtain a typical 'light' oil, a comparison was made between dark cultures and those grown in continuous light of 200, 2000 and 20,000 lx for a culture period of 4 weeks (Fig. 1). The highest growth rate was obtained in the dark. It decreased with increasing light intensity.

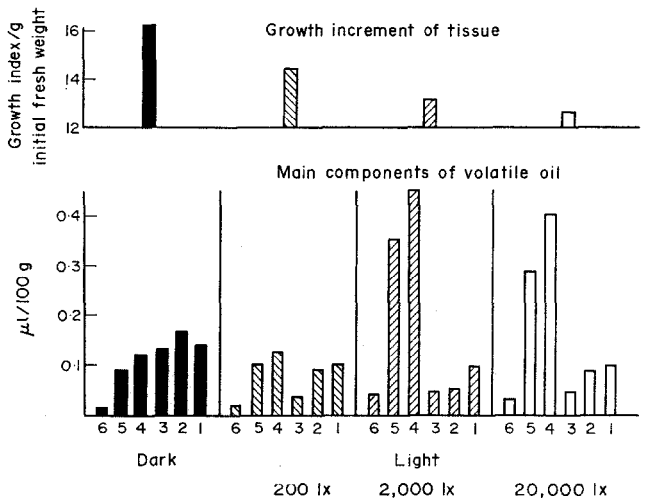

FIG. 1. GROWTH RATE AND MAIN COMPONENTS OF THE VOLATILE OIL OF AGAR CULTURES GROWN UNDER DIFFERENT LIGHT INTENSITIES.

(1)—2-nonanone; (2)—2-nonanyl acetate; (3)—2-nonanol; (4)—2-undecanone; (5)—2-undecanyl acetate; (6)—2-undecanol.

In the dark the compounds of the nonanone group (1, 2, 3) were synthesized preferably and these decrease with increasing light intensity. On the other hand, the compounds of the undecanone group increase with increasing light intensity. At a light intensity of 200 lx the shift from the 'dark' oil composition to the 'light' oil composition may already be seen. At 2000 lx the oil synthesis reaches a maximum, and the oil is composed mainly of 2-undecanone and 2-undecanyl acetate which is the typical 'light' oil composition. At 20,000 lx the composition of the oil is still the same but the total quantity is less. Hence all further experiments in light were carried out at 2000 lx.

Influence of Age on Growth and Oil Quantity

The synthesis of compounds of the so-called secondary metabolism depends on the age of the tissues during a given passage. This was demonstrated by Constabel[5] with respect to

[5] F. CONSTABEL, *Planta* **79**, 58 (1968).

the synthesis of tannins, Kaul and Staba[6] and Chen *et al.*[7] with respect to the synthesis of visnagin, and it is true for the synthesis of alkaloids in cultures of *Peganum harmala.*[8]

We therefore examined this factor in the growth of tissues and the synthesis of oil in the callus cultures of *Ruta graveolens.* Growth index and oil quantity were determined 3, 4, 5, 7 and 9 weeks after inoculation (Fig. 2). The data obtained after a culture period of 4 weeks were taken as 100% in order to allow comparison between different experiments. In the 'light' cultures the oil quantity reaches its maximum after 4 weeks, whereas the cultures continue to grow until the seventh week. In 'dark' cultures synthesis of oil continues until the sixth week. The maximum growth rate is reached after 9 weeks.

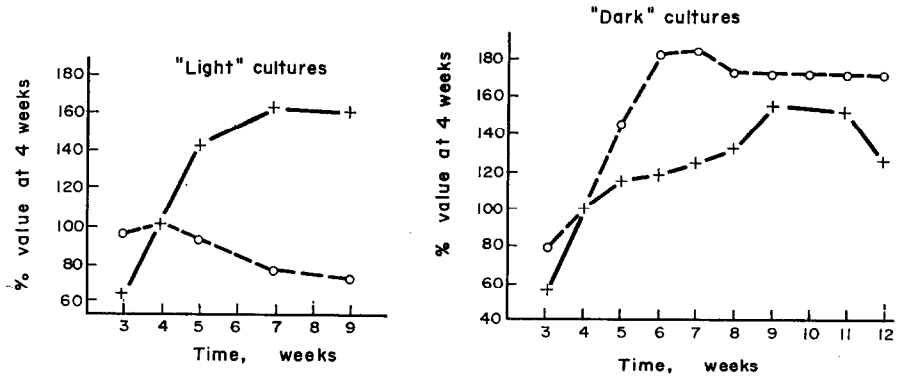

FIG. 2. GROWTH AND OIL QUANTITY IN RELATION TO AGE OF THE CULTURE.
(+)—growth; (○)—quantity of oil.

Influence of Autotrophic or Heterotrophic Conditions on Growth and Oil Quantity

To study the influence of autotrophic and heterotrophic conditions on growth and oil quantity the tissues were exposed to three different aeration systems in the light. Aeration with air served as control, allowing respiration as well as photosynthesis. In another experiment, photosynthesis was suppressed by aeration with pure oxygen. A third group of tissues was aerated with a mixture of N_2/CO_2 (99/1, v/v).

The growth and oil quantity was measured over 2 passages. After the first passage, the growth rate as well as the oil quantity was lower in O_2-enriched and O_2-deprived cultures than in the controls. After the second passage, the oil quantity was higher in the tissues aerated with O_2 (Fig. 3). The tissues aerated with N_2/CO_2 were transferred to a pure inorganic medium after the second passage. In this way we obtained autotrophic tissue cultures,[9] which could be maintained for 3 years.

The qualitative composition of the volatile oil was similar for tissues grown under heterotrophic and autotrophic conditions. In each case the typical light oil composition was observed.

Effect of Light and Dark on the Composition of the Volatile Oil

In the light mainly the high boiling compounds 2-undecanone, 2-undecanyl acetate and

[6] B. KAUL and E. J. STABA, *Planta Med.* **15,** 145 (1967).
[7] M. CHEN, S. J. STOHS and E. STABA, *Lloydia* **32,** 339 (1969).
[8] E. REINHARD, G. CORDUAN and O. H. VOLK, *Phytochem.* **7,** 503 (1968).
[9] G. CORDUAN, *Planta* **91,** 292 (1970).

2-undecanol were found. In the dark the lower boiling compounds 2-nonanone, 2-nonanyl acetate and 2-nonanol dominate. In addition, when the culture period exceeds a certain time, 2 unknown substances, detected by gas chromatography, predominate in the oil.

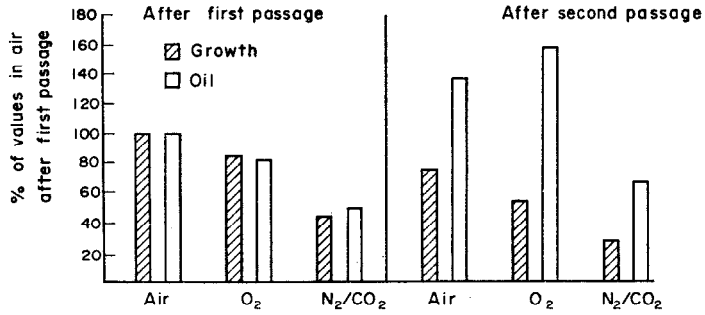

FIG. 3. INFLUENCE OF DIFFERENT GAS MIXTURES ON GROWTH AND OIL QUANTITY.

The Change of the Oil Composition from 'Light' to 'Dark' Oil

Tissue (1 g) was inoculated in light in each of the 300 ml Erlenmeyer flasks containing 100 ml agar medium. Then these flasks were put into darkness and at weekly intervals the composition of the volatile oil was analysed (Fig. 4). Until the third week the compounds of the higher boiling group, 2-undecanone and 2-undecanylacetate, still predominated in the oil. Then the oil composition shifted progressively to a typical 'dark' oil.

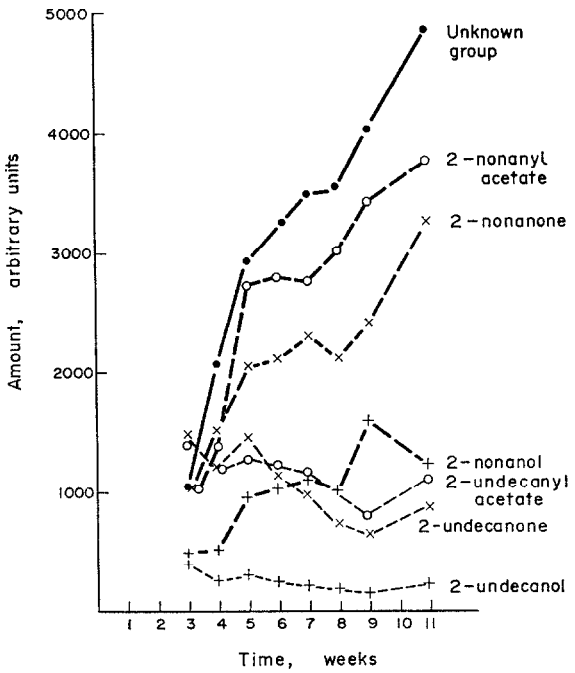

FIG. 4. THE CHANGING COMPOSITION OF THE VOLATILE OIL OF CALLUS CULTURES DURING GROWTH IN DARK.

DISCUSSION

Autotrophic and heterotrophic cultures kept in the light synthesized a typical 'light' oil. Only a transfer from light to darkness lead to a significant change in the oil composition. Gradually the quantity of the higher boiling components, mainly undecanone, decreased and the quantity of the lower boiling compounds increased. After eleven weeks in darkness the main components of the oil were two unidentified substances. The composition of this oil resembles the composition of the root oil or stem oil of the differentiated plant. Kubeczka[2] demonstrated that the oil composition of *Ruta graveolens* varies in the different organs of the plant. The composition of the leaf oil is similar to the 'light' oil of our tissue cultures. The oil of the roots, and stems covered by a bark, is different. Here a compound predominates which could be identical to one of our unknowns.

We therefore investigated the oil from roots of *Ruta graveolens* var. diwaricata collected 1969 in Yugoslavia. This oil contained two unknown compounds which represent about 70% of the volatile oil of the roots. This root oil and that obtained from tissue cultures kept in the dark for several weeks had a similar composition. On the other hand transfer of dark cultures into the light shifted the oil composition from 'dark' oil to 'light' oil, the latter resembling leaf oil of the differentiated plant. It is evident that, in the dark, the unknown compounds are synthesized preferably, whereas in the light, biosynthesis of the undecanone group predominates. In the light, compounds of the undecanone group are the main components of the oil. Their biosynthesis is evidently light-dependent. It may be pointed out that the formally related biosynthesis of fatty aids is also partly light dependent.[10,11] This light effect on oil composition does not depend on photosynthesis, since cultures grown in an O_2 atmosphere (in the presence of 10 μM DCMU) lost their green color after 3–4 weeks, but continued to synthesize the typical 'light' oil. This light effect can be modified to a certain degree by the physiological age of the cultures.

Thus the composition of the volatile oil of callus cultures of *Ruta graveolens* is light-dependent. The autotrophic or heterotrophic state of the cultures, the excretion of the oil in secretory passages or oil cells are of no influence on the oil composition. It can therefore be assumed that the composition of the volatile oil in the various organs of the differentiated plant is chiefly governed by light.

EXPERIMENTAL

Nutrition and culture conditions. The tissue cultures of *Ruta graveolens* originally obtained from stem callus of the plant were cultivated on the mineral medium of White[12] supplied with 3% coconutmilk, 20 g saccharose, 0·1 mg thiamin, 2·0 mg α-naphthyl-acetic acid, 0·2 mg kinetin and 6·5 g agar (Merck puriss.). The tissues were grown in 300 ml Erlenmeyer flasks, each filled with 100 ml agar medium. Cultures kept in the light are refered to as 'light' cultures. Normally they were maintained at 25° under continuous illumination, of 2000 lx, using 40 W fluorescent tubes (Fluora tubes, Osram). In experiments where 20,000 lx were needed, Hg-lamps were employed. The light intensity was measured outside the culture vessels at the level of the tissues. Cultures kept in the dark are refered to as 'dark' cultures. They also were maintained at 25°.

Growth measurement. The growth of the tissues after 28 days (=one passage) is expressed as the growth quotient by dividing the initial fresh weight by the final fresh weight.

Distillation of the oil. The volatile oil was obtained by steam distillation of the tissues in the Karlsruher-apparatus of Stahl.[13] For an analysis, 100 g of tissue were suspended in 250 ml water, distilled for 2 hr and collected in pentane. The volume of the pentane–oil mixture was determined and aliquots used for the gas chromatographic investigations.

[10] P. K. Stumpf and A. T. James, *Biochem. Biophys. Acta* **70**, 20 (1963).
[11] P. K. Stumpf, J. M. Bove and A. Goffeau, *Biochem. Biophys. Acta* **70**, 260 (1963).
[12] Ph. R. White, *Ann. Rev. Biochem.* **11**, 615 (1942).
[13] E. Stahl, *Pharm. Ind. (Aulendorf)* **14**, 262, 305 (1952).

For data from growth measurements, or quantitative determinations of the volatile oil, or its individual compounds, in each experiment at least four replications were made.

GLC. To separate the compounds of the volatile oil a Beckmann gas chromatograph model GT 2 A with thermal conductivity detector and a copper column (360 × 0·6 o.d. cm) filled with 20% Carbowax 20,000 on Sterchamol (mesh size 80 μ) was used. Hydrogen served as the carrier gas at a flow rate of 92 ml/min at a temperature of 160°C.

Identification and determination of the components. The six main components of the oil were qualitatively and quantitatively identified and determined by comparison with reference compounds. Undecanone and nonanone were purchased. The alcoholic compounds were obtained by reduction of the ketones with $LiAlH_4$ and acetates prepared by acetylation of the alcohols. In the case of the unknown compounds the retention times on different columns were compared. The following columns were used: 20% Carbowax 20,000, 10% high vacuum silicon grease, 15 and 20% diglycerol as well as combinations of two columns: Carbowax and high vacuum grease as well as carbowax and diglycerol. For these qualitative experiments, a Varian gas chromatograph with a flame ionization detector was used.

Key Word Index—Ruta graveolens; Rutaceae; volatile oil; synthesis; callus tissue culture.