METABOLITES OF THE PATHOGENIC FUNGUS *Verticillium dahliae*

III. THE LIPID COMPOSITION OF *Verticillium dahliae*

N. N. Stepanichenko, S. D. Gusakova, A. A. Tyshchenko,
S. Z. Mukhamedzhanov, A. U. Umarov, and O. S. Otroshchenko

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To investigate the biochemical essence of the infection process in verticilliaceous wilt of the cotton plant, we must discover the laws of the interaction of *Verticillium dahliae* Kleb. and the plant host at the level of the metabolic reactions and, in particular, of the metabolism of the fatty acids and lipids.

The symptoms of verticilliaceous wilt in the cotton plant appear during the period of fruit formation, i.e., that period of the life cycle of the plant in which the main metabolic process is concentrated on the synthesis of fatty acids and other lipids [1]. A correlation exists between the accumulation of free fatty acids during flowering and fruit-formation in the cotton plant and the resistance of the given variety to verticilliaceous wilt. A number of microorganisms produce biologically active lipid components [1, 2]. The lipids form part of the phytotoxic protein–lipid–polysaccharide complex [3]. Extracellular triglycerides are present in the culture liquid of *V. dahliae* [4]. There is information on the lipid composition of *V. albo-atrum* [5], but there is nothing in the literature on the lipid composition of *V. dahliae*.

The small amount of information on the lipid metabolism of *V. dahliae* is due to the necessity for studying all classes of lipids of the parasitic fungus and the lipid metabolism both in a pure culture and in the "plant host–parasite" system. In the present paper, we give the results of a determination of the lipid composition of *V. dahliae*.

The mycelium and the culture liquid of a 15-day growth of the fungus *V. dahliae* of the Yangiyul' population was used. The lipid fraction was isolated by the extraction of the mycelium, previously fixed with nitrogen, and the culture liquid with diethyl ether. The lipid fraction from the mycelium of *V. dahlia* consisted of a yellow oily liquid with a sharp unpleasant smell and amounted to 23% of the weight of the air-dry mycelium. To isolate the neutral lipids, the combined lipids were subjected to column chromatography (CC) on Al_2O_3. The combined neutral lipids amounted to 80% of the weight of the whole lipid fraction.

The composition of the classes of the lipid fractions was determined by thin-layer chromatography (TLC) in system 1. The quantitative ratio of the fractions was determined by a gravimetric method. The preliminary identification was performed by comparing the R_f values of the spots with literature information [6] and with the R_f values of model samples: nonacosane, methyl oleate, tristearin, β-sitosterol, and oleic acid. On chromatography, the combined neutral lipids were separated into eight zones, of which the three relatively most polar were not identified.

The composition of the neutral lipids was shown in Table 1. In the total lipid fraction, the predominant components are the triglycerides (60%) and the free fatty acids (20.2%).

We investigated three fractions with R_f 0.60, 0.57, and 0.50, respectively. They were isolated by preparative thin-layer chromatography (PTLC), were rechromatographed in system 1, and were investigated by spectral and chromatographic methods.

The mass spectrum of the fraction with R_f 0.6 (methyl esters of fatty acids) has molecular ions with m/e 296, 294, 270, and 268, which correspond to the molecular weights of the

V. I. Lenin Tashkent State University, Institute of the Chemistry of Plant Substances, Academy of Sciences of the Uzbek SSR, Tashkent. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 431-435, July-August, 1976. Original article submitted January 7, 1976.

TABLE 1

Fraction number	Class of lipids	Fluorescence in UV	R_f	Content, %
8	Carbohydrates Sterol esters	Weak bluish green	0,90	4,0
7	Methyl esters of fatty acids	None	0,60	8,0
6	Triglycerides	None	0,57	60,0
5	Free fatty acids	None	0,50	20,2
4	Not identified	None	0,33	1,0
3	Free sterols	Deep blue	0,25	2,0
2	Not identified	Pale blue	0,18	1,5
1	Not identified	Pale blue	0,00	1,2

methyl esters of oleic, linoleic, palmitic, and palmitoleic acids. These facts were confirmed by the results of spectra (IR, PMR) and chromatographic (TLC, GLC) investigations.

Spectral (PMR, IR, mass) analysis showed that the fraction with R_f 0.50 is the total free fatty acids. Methylation with diazomethane gave the corresponding methyl esters. According to GLC, the combined acids consist of $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$. The separation of the combined free fatty acids in the form of methyl esters by the PTLC + $AgNO_3$ method in system 2 and spectral analysis (UV, IR, PMR, and mass spectra) of the individual acids confirm the correctness of the identification of the free fatty acids.

The triglyceride fraction from the mycelium was isolated from 8.3 g of the lipids by the PTLC method; the triglycerides were rechromatographed and their purity was checked by TLC. This gave 5.5 g of triglycerides in the form of a pale yellow mobile oil. The parameters of the PMR and IR spectra of these fractions correspond to those given in the literature for triglycerides [7, 8].

The UV spectrum of the triglycerides has weak absorption in the 223, 264, 272, 283, 294, and 328 nm regions, which shows the presence of unidentified components.

To isolate the fatty acids, part of the triglycerides was saponified under mild conditions with the separation of the unsaponified products by extraction with diethyl ether. The fatty acids were methylated with diazomethane and investigated by chromatographic (GLC, TLC) and spectral methods.

The fatty-acid composition of the lipid fractions was as follows (%):

Acid	Methyl esters	Free fatty acids	Monoglycerides	Triglycerides
$C_{16:0}$	35.6	53.7	—	23.5
$C_{16:1}$	1.8	—	—	1.1
$C_{17:0}$	—	—	—	0.3
$C_{18:0}$	—	2.8	—	1.1
$C_{18:1}$	59.0	41.2	93.2	51.1
$C_{18:2}$	3.6	2.3	6.8	22.5

The fractions of methyl esters, free fatty acids, and triglycerides differ somewhat in their fatty-acid composition both qualitatively and quantitatively. The most complete set of acids is present in the triglyceride fraction, where the $C_{16:0}$, $C_{18:1}$, and $C_{18:2}$ acids predominate. In the methyl ester and free fatty acid fractions the predominating acids are $C_{16:0}$ and $C_{18:1}$.

The results of the GLC of the methyl esters of the combined acids were confirmed by analytical TLC on silica gel impregnated with $AgNO_3$ in benzene. Under these conditions, the methyl esters of the fatty acids are separated according to their degree of unsaturation. Five fractions were obtained corresponding in degree of decreasing R_f to saturated acids, R_f 0.95 (32.1%), monoenoic acids with R_f 0.89 (47.5%), and dienoic acids with R_f 0.74 (11.3% Fractions with R_f 0.61 (4.7%) and 0.30 (4.5%) were not identified. Gas–liquid chromatography showed that the unsaturated-acid fractions consisted of 99.7% of palmitic acid, $C_{16:0}$, and 0.3% of stearic acid, $C_{18:0}$, and the monoenoic fraction of 99.3% of $C_{18:1}$ and 0.7% of $C_{11:1}$, and the dienoic fraction was 100% linoleic acid.

To isolate the individual acids, the methyl ester fraction obtained from the triglycerides was separated by TLC + $AgNO_3$ with rechromatography, and the individual methyl esters were investigated by spectral and chemical methods.

The IR, PMR, and mass spectra of the methyl esters isolated corresponded in the main to the normal methyl esters of palmitic, oleic, stearic, linoleic, and palmitoleic acids [7-9].

In the PMR spectrum of the individual methyl esters of the unsaturated acids, a shift of the region of the resonance signal of the olefinic protons from 4.64-4.76 to 4.82 ppm was observed.

The positions of the double bonds in the $C_{16:1}$, $C_{18:1}$, and $C_{18:2}$ acids was confirmed by the destructive oxidation of the acids at the double bonds [10]. The fragments from the destruction of $C_{18:1} + C_{16:1}$ contained azelaic, pelargonic, and heptanoic acids.

To determine the types of distribution of the acyl radical in the trigylceride molecules, part of the triglyceride fraction was subjected to enzymatic hydrolysis with lipase. The hydrolyzate obtained was separated by PTLC on silica gel in system 1. In this system, the hydrolyzate was separated into seven fractions with the following R_f values: start; R_{f_1} 0.17; R_{f_2} 0.29; R_{f_3} 0.37; R_{f_4} 0.61; R_{f_5} 0.73; R_{f_6} 0.76; R_{f_7} 1. The fractions obtained were studied by spectral methods and by comparison with model samples, leading to the following assignments: R_{f_1} 0.17 — monoglycerides; $R_{f_{2,3}}$ 0.29 and 0.37 — unidentified; R_{f_4} 0.61 — free fatty acids; R_{f_5} 0.73 — aromatic ester; R_{f_6}, 0.76 — methyl esters of fatty acids; R_{f_7} 1 — carbohydrates.

The mass spectrum of the monoglyceride fraction showed molecular ions with m/e 356 and 354, which correspond to the molecular weights of monoglycerides esterified in position 2 with the $C_{18:1}$ and $C_{18:2}$ acids. The total fragmentation of the monoglyceride fraction corresponds to that described in the literature [11]. After saponification of the monoglyceride fraction, acids were isolated which were analyzed in the form of their methyl esters by the GLC method. This showed that the monoglycerides were esterified in position 2 only by the 18:1 and 18:2 acids. It must be mentioned that under the conditions used the triglycerides were hydrolyzed completely. From the results of hydrolysis we calculated the glyceride composition of the lipids (S — the saturated acids $C_{16:0}$, $C_{17:0}$, $C_{18:0}$; U — the unsaturated acids $C_{16:1}$, $C_{18:1}$, $C_{18:2}$):

Type of Triglycerides	Amount, %	Type of Triglycerides	Amount, %
Gl SSS	0,9	Gl USU	20,4
Gl SSU	11,9	Gl SUU	24,0
Gl SUS	1,8	GLH_3	41,0

Thus, the main types of trigylcerides are tri- and diunsaturated glycerides (85%).

As mentioned above, the fraction with R_f 0.73 obtained after the enzymatic hydrolysis of the triglycerides was assigned on the basis of spectral characteristics to the aromatic esters. The coloration of the spot when the chromatogram was treated with 50% H_2SO_4 followed by heating at 150°C changed on standing from pinkish brown to lilac. The mass spectra of this fraction showed a peak with m/e 278, and the UV spectrum had maxima at 225 and 260 nm (CH_3OH). The IR and PMR spectra and mass fragmentation are analogous to those described in the literature [12] and correspond to diisobutyl phthalate.

The lipid fraction from the culture liquid of *V. dahliae* consisted of a pink odorless oily liquid. Qualitative analysis of the composition of the lipids of the culture liquid showed that its main components were carbohydrates, methyl esters of fatty acids, the aromatic ester, and triglycerides. Free fatty acids and the more polar components observed in the lipid fraction from the mycelium of the fungus are absent.

The results of the analysis of the fatty-acid composition of the triglyceride fraction showed that, as in the triglyceride fraction from the mycelium, the $C_{18:1}$ and $C_{18:2}$ unsaturated acids predominate in the lipid fraction of the culture liquid. The high percentage of unsaturated fatty acids in the triglyceride, free fatty acid, and methyl ester fractions of the fungus *V. dahliae* shows a connection of their biosynthesis with the activity of the oxidative enzyme systems. There is not doubt that in the development of the pathological process when the cotton plant is infected with wilt, these factors may have a not unimportant role.

EXPERIMENTAL

The method of growing the fungus, the method for the primary treatment of the culture liquid and the mycelium of the fungus *V. dahliae*, the isolation of the neutral lipid frac-

tion, and also the conditions of recording the PMR spectra were similar to those described previously [13]. Qualitative and preparative TLC were performed on L 5/40 silica gel (Chemapol) + 10% of gypsum in the hexane–diethyl ether–acetic acid (70:30:1) system (system 1). The chromatograms previously examined in UV light were sprayed with 50% H_2SO_4 and heated to 150°C. The mixture of fatty acids was isolated and analyzed by methods described previously [14].

The IR spectra were recorded on a UR-10 spectrometer (in a film), the mass spectra on a "Massenspectrometer MAT-311," the UV spectra on a Beckman model 25 in hexane, and the NMR spectra on a Varian exel-100 instrument.

SUMMARY

The compositions of the neutral lipids of the mycelium and of the culture liquid from the pathogenic fungus *Verticillium dahliae* Kleb. have been investigated for the first time. The fatty-acid compositions of the main lipid fractions and the glyceride composition of the triglyceride fraction have been determined. The main acids of all the fractions are the 16:0 and 18:1 acids and in the triglyceride fraction, additionally, the 18:2 acid. Diisobutyl phthalate has been found in the combined neutral lipids of the fungal mycelium.

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