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LIPID COMPOSITION OF THE SEEDS OF THE COTTON PLANT INFECTED WITH WILT

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The qualitative and quantitative set of lipid components of the seeds of healthy cotton plants of the variety Tashkent-1 and plants infected with verticillium wilt have been studied. It has been established that the compositions of the lipids of the two samples differ considerably. The greatest differences are observed in the amounts of total lipids, gossypol, and low-molecular-weight volatile acids.

On prolonged permanent cultivation, the resistance of various varieties of the cotton plant to attack by the pathogenic fungus *Verticillium dahliae* Kleb. falls and the degree of injury and of the harmfulness of the disease increase [1]. The susceptibility of the plant to the disease leads not only to a reduction in yield and a deterioration of the quality of the fiber but also to a decrease in the germinating capacity and energy of growth of the seeds [2]. Although the opinion is generally accepted that the seeds of the cotton plant are the bearers of the infection, the presence of endogenous infection in them in cases of wilt damage is disputed [3]. It has been established that infected seeds of the medium-resistant variety 108-F possess a smaller weight, an undersized nucleus, and a lower oil content [4]. There is no information in the literature on the lipid composition of the seeds of the wilt-infected plant.

In the present communication we give the results of a comparative study of the neutral lipids of the seeds of a healthy cotton plant of the variety Tashkent-1 and of a plant attacked by verticillium wilt. The analysis of the lipid composition was carried out in duplicate with samples of freshly-gathered seeds of the 1977-1978 harvest. The combined lipids of the seeds of the samples mentioned of the 1978 harvest preserved the same features as in the lipids of samples from the preceding year. We studied the lipids of the seeds of the 1978 harvest.

The yields of neutral lipids from the seeds of the healthy cotton plant (sample I) and of the plants suffering with wilt (sample II) amounted to 22.5% and 15.9%, respectively. The total lipids were separated by column chromatography into individual classes of compounds. In the lipids of sample I we found hydrocarbons, triacylglycerols (TAGs), high-molecular-weight fatty alcohols, free fatty acids (FFAs), epoxyacylglycerols (EAGs), hydroxyacylglycerols (HAGs), free sterols, gossypol, and polar lipids. In the lipids of sample II, in addition to the nine classes mentioned, there were also diacylglycerols (DAGs) and monoacylglycerols (MAGs). The assignment of each fraction to a definite class of compounds was carried out on the basis of chromatographic and spectral characteristics. The compositions of the oils from the healthy and infected cotton plant according to classes were as follows (%):

	Sample I	Sample II
Hydrocarbons	0.1	0.1
TAGs	92.5	90.3
High-molecular-weight fatty alcohols	tr.	tr.
FAAs	0.8	1.1
EAGs	0.5	1.1

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	Sample I	Sample II
HAGs	2.3	Σ3.4
DAGs	—	
Sterols	1.6	1.9
Total gossypol	0.57	0.28
Free gossypol	0.48	0.23
MAGs	—	0.1
Polar lipids	1.4	1.3

As can be seen from the figures given above, in sample II the amount of TAGs was somewhat smaller, the amount of gossypol only half, and the amount of free fatty acids greater than in sample I. From the HAG fraction of sample I after acid methanolysis we isolated 14.3% of hydroxy acids, and from the combined HAG and DAG fractions of sample II 15.5% with respect to the weight of the fractions. Since the hydroxy acids are components of the HAGs but not of the DAGs, in the infected cotton plant the oxidized acylglycerols (HAGs + EAGs) are present in somewhat greater amount than in the healthy plant. Consequently, the lipid composition of the seeds of the infected cotton plant differs appreciably from the composition of the seeds of the healthy plant.

Analogous changes are observed in the lipid composition of grain crops infected with the fungus *Fusarium sporotrihiella* Bilai [5]. In the literature there are statements that in vegetative organs of the diseased cotton plant the nitrogen, protein, and carbohydrate metabolisms are disturbed [3, p. 25]. The variability of the seed lipids on the infection of the cotton plant by wilt also shows a change in the lipid metabolism. It has been established that in the process of peroxidase oxidation of the fat-soluble pigment gossypol active forms of oxygen are produced which are responsible for the toxicity of the gossypol for parasitic microorganisms [6]. It is also known that in the infection-damaged cotton plant the activity of the oxidizing enzymes is increased [7]. Consequently, the decrease in the amount of gossypol and the increase in the amount of oxidized lipids in the seeds of the infected cotton plant may be a consequence of the activation of peroxidase systems. The appearance in the infected seeds of monoacylglycerols and diacylglycerols and the increase in the amount of free acids is in harmony with the fact that in stress situations hydrolytic processes also take place more intensively in the plant [3, 8].

To evaluate all the oxygen-containing fractions we determined their fatty-acid compositions. The total amount and composition of the low-molecular-weight volatile acids were established only for the total lipid materials of the two samples.

From the results of the determination (Table 1), almost all the lipid fractions had the same set of 7-9 high-molecular-weight acids. The compositions of the free acids in the two samples differed to a considerably greater degree. In the quantitative respect, the individual lipid fractions of sample II contained 2-8% more of unsaturated acids than the fractions of sample I, mainly in relation to the 18:2 acid, while in the total lipids the situation is the opposite. A repeat analysis gave similar results. The causes of the discrepancies are

TABLE 1. Fatty-Acid Compositions of the Oxygen-Containing Fractions of the Lipids on Healthy (I) and Infected (II) Cotton Plants (%)

Acid	Total lipids		TAGs		FFAs		EAGs		HAGs		MAGs
	I	II	I	II	I	II	I	II	I	II*	II
$C_{14:0}$	0,5	0,7	1,1	1,1	1,5	1,3	0,5	0,6	0,5	1,5	0,7
$C_{15:0}$	Tr.	Tr.	—	—	Tr.	Tr.	Tr.	0,9	Tr..	Tr.	Tr.
$C_{16:0}$	27,6	28,2	27,3	24,8	35,4	34,4	18,9	10,0	24,7	26,7	37,0
$C_{16:1}$	1,7	1,8	1,3	1,3	1,0	0,5	1,5	1,4	1,9	2,1	1,0
$C_{18:0}$	1,3	2,4	2,7	2,6	1,1	—	2,5	1,8	1,3	1,8	2,3
$C_{18:1}$	16,6	18,5	18,9	19,1	37,2	31,6	17,2	19,7	40,2	27,8	23,6
$C_{18:2}$	52,3	48,4	48,7	51,1	22,4	31,4	59,4	57,9	31,4	40,1	35,4
$C_{18:3}$	Tr.	Tr.	Tr.	Tr.	1,4	0,8	—	7,7	—	—	—
$C_{20:0}$	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	—	—	—	—	—

*The combined composition of the HAGs and DAGs is given.

not clear, but a fall in the amount of the 18:2 acid in the combined acids of the total lipid material has also been observed in the artificial infection of oat grains with the fungus *Verticillium dahliae* [9].

The difference between the samples in the amount of low-molecular-weight acids was more pronounced. In the oil from the seeds of the healthy plant, the total amount of these acids was 0.17% (Reichert-Meissl No. 0.81 ml KOH) and for the diseased plant it was 0.58% (Reichert-Meissl No. 2.73 ml KOH), which is almost 3.5 times greater. The compositions of the volatile acids were determined by the thin-layer (TLC) and gas–liquid chromatography (GLC) methods. On TLC in system 3 in comparison with models, the spots of C_2 (R_f 0.33), C_3 (R_f 0.45), C_4 (R_f 0.59), C_5 (R_f 0.71), C_6 (R_f 0.83), and of the combined C_7, C_8, and C_9 acids (R_f 0.95) were detected.

According to GLC, the composition of the acids of sample I was as follows (%): C_2, 15.6; C_3, 18.6; C_4, 10.6; C_5, 7.0; C_6, 7.3; C_7, 7.8; C_8, 4.2; C_9, 28.9. The composition of the acids for sample II was (%): C_2, 21.6; C_3, 11.4; C_4, 11.4; C_5, 4.6; C_6, 9.1; C_7, 13.0; C_8, 7.2; C_9, 21.7. In sample I, consequently, the C_3 and C_9 acids predominated, and in sample II the C_2 and C_9 acids, and the amounts of the C_2, C_3, C_7, and C_9 acids also changed appreciably. The fungitoxicity of the low-molecular-weight organic acids is known. In lignified stems (wood) of the wilt-infected cotton plant of variety 108-F with an increase in the total amount of lipids by a factor of 1.5 the amount of organic acids rose threefold as compared with the healthy plant [9, p. 40]. The same authors [9] established that the organic acids of the wood of the diseased plant contained, in addition to the C_3, C_4, and C_6 monocarboxylic acids, exhibiting a high fungicidal activity with respect to the causative agent of wilt, also the inactive dicarboxylic acids $C_7H_{14}(COOH)_2$, $C_6H_{12}(COOH)_2$, and $CH_2(COOH)_2$. The reduction in the total amount of lipids in the seeds and its increase in the wood of diseased plants — one of the sites of localization of the infection — and the quantitative changes in the organic and fatty acids and gossypol in the diseased plant as compared with the healthy plant can be explained by the participation of its lipid components in protective reactions of the plant in response to infection.

It must be mentioned that when the seeds of samples I and II were stored for a year under laboratory conditions changes took place in the fatty acid composition of the total lipid material. Inboth samples the amount of 18:2 acid rose — from 52.3 to 57.1% in sample I and from 48.4 to 55.7% in II.

The compositions and structures of the TAGs of the two samples differed little from one another. The position-species compositions of the TAGs were determined by lipolytic hydrolysis. According to GLC, the acid composition of the β-MAGs for sample I obtained as the result of enzymatic hydrolysis was as follows: β-MAG-I (mol-%): $C_{16:0}$, 4.9; $C_{16:1}$, 0.9; $C_{18:1}$, 23.0; $C_{18:2}$, 71.2. Acid composition of sample II, β-MAG-II (mol-%): $C_{16:0}$, 4.5; $C_{16:1}$, 0.9; $C_{18:1}$, 24.3; $C_{18:2}$, 70.3. The position-species compositions are given below (mol-%; P, 16:0; S, 18:0; O, 18:1; L, 18:2):

Species	TAG-I	TAG-II	Species	TAG-I	TAG-II
PLL	20.8	20.8	POS	1.2	1.0
PLP	11.9	9.8	SOL	1.0	1.2
PLO	10.2	9.0	PPO	0.8	0.6
LLL	9.1	11.2	PPP	0.8	0.6
OLL	9.0	9.6	OOO	0.7	0.8
POL	7.0	7.4	OPL	0.6	0.6
POP	4.0	3.5	LPL	0.6	0.6
PLS	3.4	3.0	SOO	0.5	0.6
POO	3.4	3.2	SLS	0.2	0.2
POL	3.0	4.0	POP	0.2	0.1
OOL	3.0	3.4	PPS	0.2	0.2
SLL	3.0	3.2	SPL	0.2	0.2
OLO	2.2	2.1	SPO	0.1	0.1
SLO	1.4	1.4	SOS	0.1	0.1
PPL	1.4	1.4			

The number of position species of the TAGs in both samples was 29. The amount of each of the other theoretically possible species was less than 0.1%. Consequently, there was practically no difference in the structues of the glycerides of the two samples and the changes

in the amounts of the individual species were determined by the composition of the acids of the initial TAGs. Thus, there was 2% less of the species PLP and 2% more of LLL in TAG-II than in TAG-I.

EXPERIMENTAL

The UV spectra were taken on a Hitachi spectrometer in hexane, the IR spectra on a UR-10 instrument in a film, the PMR spectra on a JNM-4H-100/100 MHz instrument (10% solutions in CCl_4, internal standard HMDS), and the mass spectrum on a MKh-1303 spectrometer at an energy of the ionizing electrons of 40 eV.

Gas–liquid chromatography was performed on a Khrom-4 chromatograph with a flame-ionization detector and a stainless steel column filled with Chromaton N-AW-DMCS impregnated with 15% of Reoplex-400. The column was 2.5 m long and 4 mm in diameter, the temperature was 198°C, the rate of flow of carrier gas (helium) 62 ml/min, of H_2 60, and of air 60 ml/min. The volatile acids were analyzed under the same conditions but at a temperature of 130°C with a rate of flow of helium of 45 ml/min.

Analytical TLC was performed on Silufol in system 1 [heptane–methyl ethyl ketone–acetic acid (43:7:1)] [10] on silica gel in system 2 [hexane–diethyl ether (9:1)], and on cellulose in system 3 [tertiary butanol–ammonia–water (20:1:4)] [11], and preparative TLC was performed on silica gel in system 4 [hexane–diethyl ether (7:3)]. The spots on the plates were revealed with iodine vapor and by spraying with 50% H_2SO_4 followed by heating.

The selection of the plants was made by O. V. Kraterov of the G. S. Zaitsev Scientific-Research Institute of Cotton-Plant Breeding and Seed Production. The seeds were collected from 100% healthy and from 100% diseased plants.

The oils were extracted from the comminuted seeds by steeping them three times with hexane or petroleum ether (40-60°C).

The column chromatography of the total lipids was carried on Chemapol L100/160 U silica gel. Hexane or petroleum ether (40-60°C) with increasing concentrations of diethyl ether (1, 2, 3, and 50%) was used as the eluent. The eluates were collected in small portions, the elution of the various classes of compounds being monitored by TLC in system 1. Where necessary, the fractions were purified by preparative TLC in system 4 or by a repetition of column chromatography. The amount of each class, with the exception of gossypol, was determined gravimetrically.

Hydrocarbons (R_f 1.0 in system 2). White grease-like substance. Mass spectrum (135°C, 40 eV, 0.5 mA): M^+ 506, 492, 478, 464, 450, 436, 422. Consequently, the material consisted of a mixture of hydrocarbons with 30-36 carbon atoms.

Triacylglycerols (R_f 0.75 in system 1). The IR and PMR spectra corresponded to triacylglycerols of ordinary fatty acids. Alkaline hydrolysis was carried out as described in Stahl's book [12] and enzymatic hydrolysis as we have described previously [13]. The position-species composition was calculated by Coleman's method [14].

High-Molecular-Weight Fatty Alcohols (R_f 0.7 in system 1). White grease-like substance. IR spectrum, ν_{max}^{KBr}, cm^{-1}: 3600-3200 s, 2960 s, 2850 s, 1660 m, cm^{-1}. Mass spectrum (180°C, 40 eV, 0.5 mA), m/e: molecular ion absent, 392, 364 ($M^+ - 18$), 336, 308 ($M^+ - 74$) and fragments corresponding to the decomposition of alcohols [15]. On this basis it was established that the material consisted of a mixture of C_{26} and C_{28} alcohols.

Free Fatty Acids (R_f 0.6 in system 1). The IR and PMR spectra corresponded to combined fatty acids.

Epoxyacylglycerols (R_f 0.5 in system 1), positive reaction with picric acid [16]. IR spectrum, ν_{max}^{film}, cm^{-1}: 3010 m, 2970 s, 2870 s, 1745 s, 1670 w, 1465 m, 1440 m, 1380 m, 1250 m, 1200 s, 1175 s, 990 w, 845 and 885 m, 730 m. Alkaline methanolysis of the epoxyacylglycerols yielded the combined methyl esters of the acids (R_f 0.9 in system 1) and the methyl esters of the epoxy acids (R_f 0.45 in system 1). The structure of the epoxy acids isolated from the epoxyacylglycerols has been given previously [17].

Diacylglycerols and Hydroxyacylglycerols (R_f 0.4 in system 1). IR spectrum, ν_{max}^{film}, cm^{-1}: 3600-3200 s, 3010 m, 2970 s, 2920 s, 2870 s, 1725 s, 1475 m, 1380 m, 1250 m, 1170 s, 1100 m, 980 and 990 w, 720 m. PMR spectrum τ, ppm: 9.15 t, 8.78 m, 8.47 m, 8.01 m, 7.75 t, 7.29 m, 6.45 m, 5.97 and 5.88 combined m, 5.11 and 4.99 combined m, 4.74 m. After alkaline

hydrolysis and methylation of the acid fraction, the combined normal esters and oxidized esters, having a chromatographic mobility similar to that of methyl ricinoleate, were obtained.

Free Sterols (R_f 0.35 in system 1). They gave a red coloration on being sprayed with 50% H_2SO_4 followed by heating. They were purified by recrystallization from methanol, mp 130-132°C. Mass spectrum (125°C, 40 eV, 0.5 mA), m/e: 414 M^+, 412 M^+, 400 M^+, 397, 395, 396, 385, 382, 369, 367, 351, 329, 315, 314, 303, 289, 275, 273, 271, 255, 231, 213. According to these facts, the combined material consisted of a mixture of β-sitosterol, α-sitosterol or stigmasterol, and kampesterol.

Monoacylglycerols (R_f 0.09 in system 1). IR spectrum, ν_{max}^{film}, cm^{-1}: 3600-3200 s, 3010 m, 2960 s, 2940 s, 2850 s, 1745 s, 1470 m, 1380 m, 1250 w, 1180 m, 1100 m, 1070 m, 920 m, 730 m. Mass spectrum (160°C, 40 eV, 0.5 mA), m/e: 299, 327, 325, 323 ($M^+ - 31$), 295, 323, 321, 319 ($M^+ - 35$), 270, 298, 296, 294 ($RCOOCH_3$), 257, 285, 283, 281 ($RCOOH+H^+$), 240, 268, 266, 264 [18].

After alkaline hydrolysis, a mixture of normal fatty acids was obtained which was methylated and analyzed by GLC.

Gossypol (R_f 0.2 in system 1). UV spectrum, $\lambda_{max}^{C_2H_5OH}$, nm: 236, 283, 289, 376. Since gossypol was not desorbed completely from the silica gel used for separating the combined lipids into classes, its amount was determined in the total lipids.

The quantitative determination of the total and free gossypol was carried out with p-anisidine [19] and by the spectrophotometric method, using the absorption maxima of ethanolic solutions of gossypol at 236 and 378 nm [20].

Low-Molecular-Weight Acids. Their percentage amount was calculated from the Reichert-Meissl number, which was determined by the usual method [21]. The acids were isolated in the form of the potassium salts formed in titration during the determination of the Reichert-Meissl number. An aqueous solution of the salts was evaporated with the addition of benzene and methanol in a rotary evaporator to a volume of 10-15 ml at 70-80°C. The residue was extracted with 15% HCl, and the acids were extracted in the presence of NaCl with diethyl ether five times. The combined extracts were washed free from HCl with aqueous salt solution. The ether was distilled off at a low temperature under a weak vacuum. Part of the volatile acids isolated was methylated with diazomethane for GLC analysis and another part was used in the form of ammonium salts for TLC in system 3 [11].

SUMMARY

It has been established that the lipid composition of the seeds of the cotton plant infected with verticillium wilt differs from that of the seeds of the healthy plants not only by a smaller content of total lipids but also by a reduction in the amount of gossypol and an increase in the amounts of oxidized forms of lipids and of free fatty acids.

The appearance of monoacylglycerols and diacylglycerols in the lipids of the seeds of the infected cotton plant has been observed.

It has been found that in the total lipids of the seeds of the diseased cotton plant the amount of low-molecular-weight organic acids is considerably increased.

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PHOSPHOLIPIDS OF THE SEEDS OF THE HEALTHY AND THE WILT-AFFECTED COTTON PLANT

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A comparative study has been made of the phospholipid complexes of the seeds of a healthy cotton plant of the variety Tashkent-1 and one attacked by verticillium wilt. Some differences have been found in the amounts of the components of the total phospholipids and in the distribution of the fatty acids in the individual fractions of the phospholipids of the healthy and wilt-affected plants.

In a comparative investigation of the phospholipids (PLs) of the seeds of a healthy cotton plant of variety Tashkent-1 and one attacked by verticillium wilt, we have confirmed that there is a far smaller amount of gossypol in the seeds of the diseased plant. There are statements in the literature [1] that resistance to wilt is connected with the accumulation of gossypol in the cotton plant. Varieties resistant to wilt are attacked in time as a consequence of the broad possibilities of adaptation of the pathogen. These varieties actively react to the introduction of the parasite by an activation of various links of the metabolism. Bell [2] considers that the main fungitoxic substance in relation to the fungus is gossypol, and therefore a decrease in its amount in the seeds of the diseased plant may probably be connected with the consumption of gossypol in combatting the fungus.

On isolating the total PLs, it was found that their yield from diseased seeds was higher than from healthy seeds (1.8% as compared with 1.5%).

The qualitative and quantitative compositions of the total phospholipids were determined by two-dimensional thin-layer chromatography. It was established that the sets of PLs from the seeds of healthy and diseased cotton plants did not differ qualitatively from one another but there were differences in the quantitative distribution of the individual components in the total.

The quantitative distribution of the individual components in the total PLs of cotton seeds of the variety Tashkent-1 were as follows (% on the total PLS; X_1 and X_2 are unidentified PLs):

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