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METABOLITES OF THE PATHOGENIC FUNGUS Verticillium dahliae

VI. PENTAKETIDE METABOLITES AND NEUTRAL LIPIDS OF VIRULENT AND AVIRULENT STRAINS

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We have previously reported the isolation of phytotoxic metabolites and their action on isolated pea chloroplasts and on the permeability of synthetic phospholipid membranes [1-3] and also the composition of the extracellular (EL's) and intracellular (IL's) lipids of the fungus Verticillium dahliae Kleb. [4, 5]. In the present paper we give the results of a comparative study of the relative amounts of phytotoxic pigment from the culture liquid (PKZh-1) and the EL's and IL's in five strains and mutants of V. dahliae differing in virulence and in the nonpathogenic fungus V. lateritium when they are grown under stationary conditions.

We investigated the KhL-1,3 and KhL-1,7 strains of V. dahliae, the mutants R-196, R-101, and S-1, and V. lateritium [6]; for comparison we used information on the amounts of PkZh-1, EL's, and IL's in the wild Yangiyul' strain L-1: (see Table on following page)

As was found, PKZh-1 was present in the culture liquid of all the virulent strains, its amount being a maximum in KhL-1,3. This phytotoxic metabolite was not present in the nonpathogenic species, V. lateritium and the avirulent mutants, which shows a definite correlation between virulence and the amount of PKZh-1

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Fungus	Strain and mutant	Virulence to the cottonplant	PKZh-1, mg/l	EL's, mg/l	IL's, g/g myc.
V. lateritium		Nonpathogenic	None	1,0	0,001
V. dahliae	KhL-1,3	Virulent to variety 108-F (race 1)	1,4	9,9	0,43
	KhL-1,7	Virulent to variety 108-F (race 2)	1,0	6,7	0,42
	R-196	Avirulent to varieties 108-F and Tashkent-1	None	1,0	0,42
	R-101	Avirulent to varieties 108-F and Tashkent-1	None	None	0,38
	S-1	Feebly virulent to variety 108-F	0 2	None	0,45
	S-1	Virulent to variety 108-F	1,2	7,2	0,35

Characteristic for the virulent strains of V. dahliae is the formation of a relatively large amount of EL's. In the avirulent mutants, no appreciable amounts of EL's were formed, but in an ether extract of the culture liquid of one of them (R-196) substance (I) was present in appreciable amounts (0.1 mg/ml). The compound isolated consisted of light grey crystals with mp 166-168°C (ethanol). On the basis of its elementary composition and spectral characteristics (UV, IR, NMR, and mass spectra), (I) was identified as 3,6,8-trihydroxy-1,2,3,4-tetrahydronaphthalen-1-one [8]. The ^{13}C NMR spectrum of (I) (in methanol) corresponded to the structure given. On the basis of the influence of the oxygen atoms in this molecule and the position of the resonances of the ^{13}C nuclei, all ten signals were assigned to the carbon atoms in (I) in the following way (chemical shifts in ppm): 201.2 – C_1; 165.8 – C_8; 165.6 – C_6; 144.8 – C_9; 110.5 – C_{10}; 108.5 – C_7; 100.8 – C_5; 66.0 – C_3; 46.3 – C_2; 38.2 – C_4.

On growth in the stationary stage under the same conditions of cultivation, the strains of the fungus V. dahliae investigated did not differ appreciably in their content of IL's, while the amount of IL's in V. lateritium was considerably smaller (see above).

The qualitative and quantitative compositions of the neutral lipids (NL's) of the mycelia of all these samples studied, determined by thin-layer chromatography under the conditions described previously [5], are given in Table 1.

The classes of lipids were identified on the basis of a comparison of the R_f values of the spots with literature information [9] and with the R_f values of model samples: nonacosane, methyl oleate, tristearin, oleic acid, and β-sitosterol. The sum of the neutral lipids of all the samples was separated on chromatography into eight zones, three of which were not identified (see Table 1). It can be seen from Table 1 that qualitatively all the samples investigated except L-1 contained no methyl esters of fatty acids (ME's), while their amount in the Yangiyul's strain was 8%. In all the samples triglycerides (TG's) and free fatty acids (FFA's) predominated. The correctness of the identification was conformed by a spectral analysis (NMR, IR, and mass spectra of the fractions isolated. By preparative thin-layer chromatography from the total lipids of all the samples we isolated the PG's and by their hydrolysis we obtained the combined fatty acids (FA's). The acids were analyzed by the GLC method in the form of methyl esters after methylation with diazomethane. The composition of the FA's was as follows (%):

Acid	*V. lateritium*	KhL-*1,3*	KhL-*1,7*	R-*196*	S-*1*	L-*1*
$C_{14:0}$	0,4	—	—	—	—	—
$C_{16:0}$	28,2	24,7	27,3	24,0	28,5	23,5
$C_{16:1}$	1,4	0,8	2,2	1 7	1,6	1,1
$C_{17:0}$	—	—	—	—	—	0,3
$C_{18:0}$	5,4	3,8	6,3	5,2	2,5	1,1
$C_{18:1}$	54,8	51,5	48,7	53,5	48,5	51,5
$C_{18:2}$	9,7	19,2	15,5	15,6	18,9	22,5
$\frac{\Sigma \text{ unsat}}{\Sigma \text{ sat}}$	1,9	2,5	1,9	2,3	2,2	2,8

Qualitatively, the compositions of the fatty acids of the PG fraction of the strains and mutants studied differ considerably. The acids of the PG's of V. lateritium contain the $C_{14:0}$ acid (0.3%), which is not synthesized by V. dahliae, and the fatty acids of the PG's of the wild strain L-1 contain the $C_{17:0}$ acid, which is not found in V. lateritium.

The maximum formation of oleic acid, $C_{18:1}$, (54.8%) was found in V. lateritium, while the amount of linoleic acid, $C_{18:2}$, in this fungus is considerably smaller (9.7%) than in the wild strain L-1 (22.5%). The differences in the fatty-acid compositions of the FFA fractions of V. dahliae and V. lateritium are still more

TABLE 1. Compositions of the Neutral Lipids of the Mycelium of Virulent and Avirulent Strains and Mutants of Verticillium dahliae and V. lateritium

Fraction	Class of lipids	R_f	Amount, %						
			V. lateritium	KhL-1,3	KhL-1,7	R-196	R-101	S-1	L-1
8	Hydrocarbons, sterol esters	0,90	2,1	0,4	1,0	0,5	0,8	1,6	4,0
7	Methyl esters of fatty acids	0,60	—	—	—	—	—	—	8,0
6	Triglycerides	0,57	74,6	82,4	72,0	75,1	70,6	71,6	60,0
5	Free fatty acids	0,50	22,3	13,0	13,0	13,6	20,6	7,8	20,2
4	Unidentified, X_1	0,33	0,2	1,6	4,0	6,1	5,1	6,4	3,0
3	Free sterols	0,25	0,5	0,8	3,0	0,7	0,9	4,6	2,0
2	Unidentified, X_2	0,18	0,2	0,2	1,0	0,3	0,4	1,6	1,5
1	Unidentified, X_3	0,00	0,1	1,6	6,0	3,7	1,6	6,4	1,2

considerable; they are shown below (%):

Acid	V. *lateritium*	KhL-*1,3*	KhL-*1,7*	R-*196*	S-*1*	L-*1*
C_{X_1}	0,9	—	—	—	—	—
C_{X_2}	23,2	—	—	—	—	—
Iso-$C_{16:0}$	0,7	—	—	—	—	—
$C_{16:0}$	38,0	24,5	40,0	19,2	41,3	53,7
$C_{16:1}$	—	2,0	5,0	2,4	4,4	—
$C_{18:0}$	5,2	—	1,8	6,9	6,1	2,8
$C_{18:1}$	31,6	25,0	22,9	24,6	38,9	41,2
$C_{18:2}$	—	48,5	30,3	43,5	9,3	2,3
$C_{18:3}$	—	—	—	3,4	—	—
$\frac{\Sigma \text{unsat}}{\Sigma \text{sat}}$		3,0	1,3	2,8	1,1	0,7

In the case of the mutant R-101, in view of the small yield of total IL's their fatty-acid compositions were determined after the alkaline hydrolysis of the total lipid fraction. Analysis by gas chromatography of the products of the hydrolyzate in the form of methyl esters showed the presence of (%): $C_{14:0}$ – 0.75; $C_{16:1}$ – 25.0; $C_{18:0}$ – 8.16; $C_{18:1}$ – 39.0, and $C_{18:2}$ – 24.4. The FFA's of V. lateritium contain, in addition to the main acids, an iso-$C_{16:0}$ acid and two unidentified acids, C_{X_1} and C_{X_2} (23.2%). These acids, and also $C_{16:1}$ acid are not present in the wild strain L-1, but $C_{16:1}$ is contained in the FFA fractions of the other strains of V. dahliae.

Thus, in the growth of V. dahliae and V. lateritium on Czapek–Dox medium under the same conditions the fungi studied differed considerably in their content of IL's. The results obtained witness qualitative and quantitative differences in the classes of lipids and in the fatty-acid composition of the fractions of the various strains, which is connected with the nature of the lipid metabolism and is determined by genetic factors which are also responsible for the virulence of the strain.

EXPERIMENTAL

The conditions for recording the UV, IR, NMR, and mass spectra were similar to those described previously [5].

The strains KhL-1,3, KhL-1,7, R-196, S-1, and R-101 obtained in the laboratory of the genetics of the immunity of the cotton plant of the Branch of General Genetics of the Cotton Plant of the Academy of Sciences of the TadzhSSR were investigated. The fungi V. dahliae and V. lateritium were grown under stationary conditions in the dark at 26-28°C on Czapek–Dox medium for 15 days. The phytotoxic pigment PKZh-1 was isolated by means of a scheme developed previously [7], and the EL's by a method which we have also described previously [4]. The lipid fraction from the mycelium was extracted and chromatographed under the conditions of our previous work [5].

Isolation and Identification of 3,6,8-Trihydroxy-1,2,3,4-tetrahydronaphthalen-1-one. The culture liquid (10 liters) was separated from the mycelium, centrifuged, and extracted with diethyl ether (2 × 1.5 liters) and, after acidification with hydrochloric acid to pH 2.0-2.5, with ethyl acetate (2 × 1.0 liter). The ethereal extract was washed with acidified water, dried over Na_2SO_4, and evaporated in vacuum. The extracts obtained were combined (yield 1.2 g). To purify (I), from accompanying pigments we used column chromatography on alumina with elution by ether and methanol successively. The substance obtained after the evaporation of the

ethereal solution was recrystallized from 95% ethanol, giving 1.0 g of (I) with mp 166-168°C, mol. wt. 194, composition $C_{10}H_{10}O_5$.

UV spectrum, λ_{max} (in ethanol): 218 shoulder, 220, 230, 285, 315 shoulder (log ε 3.96, 3.97, and 4.00, respectively). IR spectrum (KBr), ν_{max}: 3250 (broad), 2940, 2865, 1635, 1595, 1555 (w), 1490, 1420 (w), 1375, 1350, 1275, 1200, 1175, 1140, 1120, 1085, 1050, 915 (w), 850, 785, 755, 725 (w).

Mass spectrum (80°C): m/e (%) 194 (M^+, 40), 177 (10), 176 (66), 151 (21), 150 (100), 149 (25), 148 (19), 137 (12), 136 (10), 135 (12), 97 (17), 69 (35), 44 (35).

PMR spectrum (in CD_3OD), ppm: 2.6-3.2 (4H, multiplet), 4.2 (1H, multiplet), 6.0 (1H, doublet, J = 2.5 Hz), 6.2 (1H, doublet of doublets (J = 2.5 Hz, J = 0.4 Hz), 12.4 (1H, singlet).

SUMMARY

A comparative study of the amounts of the phytotoxic pigment PKZh-1 and of extracellular and intracellular lipids in five strains and mutants of Verticillium dahliae differing in virulence and in the nonpathogenic fungus V. lateritium has been made. A definite correlation has been found between virulence and PKZh-1 content.

It has been established that the extracellular lipids are produced in considerable amounts by the virulent strains while in the avirulent mutants and in V. lateritium they are absent or present in only very small amount. The contents of neutral lipids in the mycelia of strains and mutants differing in virulence do not differ significantly. There are definite differences in the compositions of the neutral lipids and in the fatty-acid compositions of the individual classes of lipids.

From the culture liquid of one of the avirulent strains (R-196), 3,6,8-trihydroxy-1,2,3,4-tetrahydronaphthalen-1-one has been isolated.

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