Chemistry of Natural Compounds, Vol. 33, No. 2, 1997

POST-INFECTIONAL CHANGES IN THE PHOSPHOLIPIDS OF THE LEAVES OF A COTTON PLANT VARIETY RESISTANT TO *Verticillium dahliae*

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UDC 547.915:677.21.632.4

The class and fatty acid compositions of the phospholipids (PLs) of young leaves of healthy cotton plants of the wilt-resistant variety 175-F and of plants of the same variety infected with the pathogenic fungus Verticillium dahliae *Kleb. have been determined. The positional distributions of the fatty acids in the main classes of PLs have been established, and their possible molecular compositions have been calculated. The changes in the composition of the PLs taking place under the influence of infection have been elucidated.*

There is information in the literature on the PLs of the leaves of some higher plants [1-3]. Only isolated studies have been devoted to the investigation of the leaves of the cotton plant developing under normal [4] and stressed [5] conditions. Verticillium wilt, caused by the pathogenic fungus *V. dahliae*, is one of the main diseases of the cotton plant and leads to a considerable loss of yield [6]. There is no information in the available literature on the influence of the infection of leaves with the phytopathogen *V. dahliae* on the composition and structure of the PLs.

Continuing an investigation of the complex of the lipids of the leaves of the cotton plant *Gossypium hirsutum* L. [7], we have made a comparative study of the PLs of young leaves of the wilt-resistant variety 175-F from healthy plants (sample I) and from plants infected with *V. dahliae* (sample II).

Extracts of lipids from the leaves were obtained by Folch's method. The total phosphorus contents of samples I and II were 0.34 and 0.24%, while the lipid phosphorus amounted to 0.30 and 0.25% (on the weight of the extract), respectively. The extracts were colored brown because of the presence of pigments of phenolic nature coextracted with the lipids [7].

The extracts were separated by countercurrent distribution into neutral and polar lipids. The polar lipids were first fractionated by CC, which permitted the elimination of glycolipids and the bulk of the pigments. Subsequent preparative TLC in systems 1, 4, and 5 gave the main homogeneous classes: phosphatidylcholines (PCs, R_f 0.33, syst. 1), phosphatidylinositols (PIs, R_f 0.42, syst. 4) and phosphatidic acids (PAs, R_f 0.05, syst. 1). On TLC in system 1, the chromatographic mobility of phosphatidylglyerols (PGs, R_f 0.37) differed little from that of phosphatidylethanolamines (PEs, R_f 0.41), and therefore for their preliminary separation we used silica gel impregnated with a 0.15 M solution of $(NH_4)_2SO_4$ [8] and system 5. Under these conditions the PGs had R_f 0.61 and the PGs 0.16. A minor class – diphosphatidylglycerols (R_f 0.85, syst. 4) – was also identified.

The structures of the individual classes of PLs were confirmed by an investigation of the water-soluble products of their acid hydrolysis and the organosoluble products of their alkaline hydrolysis and enzymolysis, and with the aid of IR spectroscopy. The results of the analysis of the PLs are given in Tables 1-3.

As can be seen from Table 1, at the same qualitative composition of the PLs the absolute level of the total PLs in sample I was 1.3 times higher than in II. The main classes were PCs and PGs, amounting to more than 2/3 of the total mass of the PLs, which agrees with figures in the literature [4]. In the infected leaves the amount of PCs, PIs, and PAs was 1.5-2 times lower, while the levels of PGs and PEs were almost the same.

Homogeneous classes of PL were subjected to mild alkaline deacylation, and the fatty acids (FAs) were isolated in the form of methyl esters, which were analyzed by GLC.

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 0009-3130/97/3302-0156$18.00

TABLE 1. Compositions and Levels of Phospholipids in the Leaves of Healthy and *V. dahliae*-Infected Cotton Plants

Class of phospholipids	Sample I		Sample II	
	mg/g a.d.w.	% on the weight of phospholipids	mg/g a.d.w.	% on the weight of phospholipids
Phosphatidylcholines	7.7	45.4	5.2	40.1
Phosphatidylglycerols	4.0	23.4	3.8	29.0
Phosphatidylethanolamines	1.9	11.4	2.2	17.0
Phosphatidylinositols	1.9	11.1	0.9	7.3
Phosphatidic acids	1.5	8.7	0.9	6.6
Diphosphatidylglycerols	Tr.	Tr.	Tr.	Tr.
Σ phospholipids	17.0	100	13.0	100

To investigate the positional distributions of the FAs in the main classes of PLs we carried out enzymatic hydrolysis of the PCs, PEs, and PGs. The FAs were isolated from the enzymolysis products and were also investigated by GLC (Table 2).

Almost all the classes of PLs of both samples had the same qualitative set of FAs, with the 16:0 and 18:3 acids predominating (Table 2). Exceptions were PGs I and II, where, together with the 16:0 acid, the 16:1 acid predominated. It is known that the PGs of the leaves of higher plants are characterized by the presence of hexadec-*trans*-3-enoic acid, 16:1(3E), which some authors consider to be a component of the photosynthetic apparatus of higher plants [9]. Moreover, the opinion has been expressed that it is, in fact, in the PGs that the formation of this acid may take place, through esterification of the 16:0 acid at the sn-2 position of the PG molecule, with its subsequent Δ3-desaturation to the 16:1 acid [10].

On a GLC chromatogram of the methyl esters of the acids of the PGs, the 16:1(3E) peak had a retention time of 1.12 (relative to 16:0), while on chromatograms of esters of the acids of the other PLs its value for 16:1(9Z) was 1.17.

The presence of the 16:1(3E) isomer was confirmed by the mass spectrometry of the methyl esters of the FAs from PGs I and II by the Ag^+-TLC method. The spectrum contained the peaks of fragments with *m/z* 236 $[M - 32]^+$ and 194 $[M - 74]^+$, which are characteristic for the breakdown of this isomer [11]. According to Ag^+-TLC in system 7, the methyl esters of the PG FAs consisted of five fractions, belonging to the saturated (R_f 0.93), monoenic 16:1(3E) (R_f 0.83) and 18:1(9Z) (R_f 0.69), dienic (R_f 0.57), and trienic (R_f 0.32) acids with chain lengths of 16 and 18 C atoms.

In the acids of PGs II, the level of 16(3E) was 1.7 times lower than in the PGs I (see Table 2) which indicates a negative influence of infection on the photosynthetic apparatus of the cotton plant.

The PCs of sample I contained the largest amount of the 18:3 acid (55%), as a result of which this class was the most unsaturated.

On analyzing the distribution of the FAs over the sn-1 and sn-2 positions of the molecules of the individual PLs (see Table 2) it may be noted that in the PLs of sample II the law of the preferential nature of the localization of unsaturated FAs, including 16:1(3E), in the sn-2 position of glycerol that is known for the PLs of healthy tissues [12] was retained. At the same time, the PEs II contained more molecular species with the 18:3 acid in the sn-2 position than the PEs I.

In all classes of PLs of the infected leaves, but particularly the PGs and PAs, a higher level of the 16:0 acid was observed in comparison with the analogous classes of healthy leaves, while the changes in the levels of individual unsaturated FAs depended on the structure of the PLs: in the PGs and PIs of sample II the amount of the 18:2 acid, in the PIs the amount of the 18:1 acid, and in the PCs the amount of the 18:3 acid had fallen sharply in comparison with the analogous PLs of sample I.

There are facts indicating that the 18:3 acid is biosynthesized by the successive desaturation of the 18:1 acid after the latter has formed an ester with the sn-2-hydroxyls of the PCs [10]. It is not excluded that the fall in the level of the 18:3 acid in PCs II is due to a disturbance of the biosynthesis of this acid as a consequence of wilt infection.

Possible position-species compositions of the PCs, PEs, and PGs were calculated from the figures of Table 2 by Coleman's method [13] (see Table 3). Table 3 does not include molecular species of the PLs the levels of which in all classes were less than 1%. It can be seen from Table 3 that infection with *V. dahliae* affects both the qualitative and the quantitative compositions of the molecular species of the PLs. Thus, the phospholipid classes of samples I and II included: 19 and 16 molecular species, respectively, for the PCs, 24 and 10 for the PGs, and 25 and 25 for the PEs. In the PCs I the 16:0-18.3 and 18:3-18:3 species predominated, and in the PCs II almost half the weight consisted of the 16:0-18:3 species. A redistribution of the levels in the direction of an appreciable increase in the number of molecules of the 16:0-18:3 species in the PEs and of the 16:0-16:0 species in the PGs is observed.

TABLE 2. Compositions and Positional Distributions of the Fatty Acids in the Leaves of Healthy (I) and *V. dahliae*-Infected (II) Cotton Plants of the 175-F Variety (%, GLC)

Methyl ester of the acid	Phosphatidylcholines				Phosphatidylethanolamines				Phosphatidylglycerols				Phophatidyl-inositols		Phosphatidic acids	
	total acids		acids in the sn-2 position		total acids		acids in the sn-2 position		total acids		acids in the sn-2 position		total acids		total acids	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
14:0	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	1.5	1.3	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	0.8	Tr.
16:0	37.7	43.5	21.2	21.4	51.8	56.1	38.3	27.5	52.8	71.5	38.6	53.9	50.0	53.1	36.4	53.5
16:1	0.4	0.9	0.5	Tr.	1.7	1.5	1.9	2.6	24.7*	14.2*	32.6*	20.6*	1.5	1.7	5.3	1.7
18:0	Tr.	5.0	Tr.	Tr.	4.3	1.5	5.2	3.2	1.9	3.4	2.8	5.7	2.1	4.4	2.2	9.2
18:1	1.9	2.0	2.3	Tr.	4.7	3.3	7.1	6.0	4.2	3.0	6.0	6.3	2.0	3.2	10.2	1.1
18:2	5.2	8.7	13.8	12.4	14.0	12.4	18.7	19.3	8.2	2.6	12.3	6.0	20.7	7.7	21.8	6.6
18:3	54.8	39.9	62.2	66.2	23.5	25.2	27.3	40.1	8.2	5.3	7.7	7.5	23.7	29.9	23.3	27.9
Σ_{sat}	37.7	48.5	21.2	21.4	56.1	57.6	43.5	30.7	54.7	74.9	41.4	59.6	52.1	57.5	38.6	62.7
Σ_{unsat}	62.3	51.5	78.8	78.6	43.9	42.4	56.5	69.3	45.3	25.1	58.6	40.4	47.9	42.5	61.4	37.3

* 16:1 (3E).

TABLE 3. Molecular Compositions of the Phospholipids with Account of Positional Isomerism

Molecular form	Phosphatidyl-cholines		Phosphatidyl-ethanolamines		Phosphatidyl-glycerols	
sn-1/sn-2	I	II	I	II	I	II
16:0/14:0	–	–	1.1	1.2	–	–
16:0/16:0	11.1	15.9	27.7	24.4	28.6	51.2
16:1/16:0	+	+	+	+	4.8	3.7
18:0/16:0	–	1.5	+	+	+	–
18:1/16:0	–	1.0	1.3	+	2.4	–
18:2/16:0	–	+	2.0	+	3.0	–
18:3/16:0	10.1	2.5	9.0	2.8	3.0	–
16:0/16:1	+	–	1.4	2.4	24.3	19.5
16:1/16:1	+	–	+	+	4.1	1.4
18:1/16:1	+	–	+	+	2.1	–
18:2/16:1	–	–	+	+	2.4	–
18:3/16:1	+	–	–	+	2.4	–
16:0/18:0	–	–	3.8	2.9	2.1	5.4
18:3/18:0	–	–	1.2	+	+	–
16:0/18:1	1.2	–	5.1	5.3	4.4	6.0
18:3/18:1	1.1	+	1.7	+	+	–
16:0/18:2	7.3	9.2	13.6	17.1	9.2	5.7
16:1/18:2	+	+	+	+	1.5	+
18:3/18:2	6.6	1.5	4.5	1.8	+	–
16:0/18:3	32.9	49.0	19.8	35.6	5.7	7.1
16:1/18:3	+	1.2	+	–	+	–
18:0/18:3	+	4.9	–	1.1	+	–
18:1/18:3	–	3.1	+	+	+	–
18:2/18:3	+	2.6	1.4	1.3	+	–
18:3/18:3	30.0	7.6	6.4	4.1		–

We have shown previously [7] that among the molecular species of the TAGs of sample I there is a higher proportion of species with 16:0 in the sn-2 position, which shows the prokaryotic type of their synthesis, while in the TAGs II there is a higher proportion of species with 18:2 in the same position, these being characteristic for the eukaryotic type of biosynthesis.

The positional species composition of the PLs permits the conclusion that in the PCs and PEs of both samples there were molecules with the eukaryotic and the prokaryotic, and in the PGs molecules predominantly with the prokaryotic, type of FA pairing.

The type compositions of the individual classes of PLs were calculated from the results on their position-species compositions. The PCs of sample I consisted of equal amounts of saturated–saturated (SS) and unsaturated–saturated (US) types, but saturated–unsaturated (SU) types predominated in both the PEs and the PGs. When the cotton plant was infected, the levels of the UU and US types fell in all classes of PLs, and, with the exception of the PGs, the proportion of US types rose.

Thus, on infection of the cotton plant with the pathogenic fungus *V. dahliae* in the leaves the levels of total and lipid phosphorus fall and those of the main lipid classes, PCs and PIs, decrease somewhat, while the amount of PEs increases; in the main classes of PLs the levels of the 16:0, 16:1(3E) and 18:2 acids change most significantly.

EXPERIMENTAL

IR spectra were taken on an IR-10 instrument in a film; mass spectra on a MKh-1321 instrument with direct injection of the sample at an ionizing energy of 70 eV, a collector current of 3 A, and a temperature of the ionization chamber of 160°C. GLC was conducted under the conditions described in [14].

For CC we used type L 100/250 μm silica gel (Czech Republic). The lipids were eluted successively with chloroform, with mixtures of chloroform and methanol in which the proportion of the latter was gradually increased from 5 to 50%, and with pure methanol.

TLC was conducted on glass plates (6 × 6, 6 × 12, 14 × 18, and 20 × 20 cm) with type L 5/40 μm silica gel (Czech Republic) containing 6.5% of added gypsum in the following solvent systems (v/v) – for one-dimensional chromatography: 1) chloroform–methanol–25% ammonia (65:25:5); 2) isopropanol–25% ammonia–water (5:4:1); 3) *n*-propanol–25% ammonia–water (6:3:1); 4) chloroform–acetone–methanol–acetic acid–water (6:8:2:2:1); acetone–ben-

TABLE 4. Type Compositions of the Classes of Phospholipids of Healthy and *V. dahliae*-Infected Cottonplant Leaves

Types	Phosphatidylcholines		Phosphatidylethanolamines		Phosphatidylglycerols	
	I	II	I	II	I	II
SS	10.9	16.8	30.8	27.4	28.7	55.6
SU	40.5	62.0	37.6	58.3	40.8	37.7
UU	38.3	16.6	17.4	9.7	17.8	2.7
US	10.3	4.6	14.2	4.6	12.7	4.0

zene–water (91:30:8); and 6) benzene; and, for two-dimensional chromatography: 7) chloroform–methanol–25% ammonia (16:6:1) (direction I) and chloroform–methanol–acetic acid–water (30:10:1:1) (direction II).

For analysis we took healthy leaves and leaves with chlorotic spots from plants in the phase of the development of three or four true leaves. The growth conditions of the cotton plants, the artificial infection of the plants with the phytopathogen, and the isolation of the cell lipids were similar to what is described in [7].

Countercurrent distribution was performed as in [15].

The amounts of total and lipid phosphorus were determined colorimetrically as in [16], the optical densities of the solutions being measured on a FÉK-M photocolorimeter.

The PLs were identified from qualitative reactions, from literature information on the chromatographic mobilities of the individual classes [15], and from IR spectra [17].

Acid hydrolysis was carried out in accordance with [18]. In the water-soluble hydrolysis products from the PIs we detected glycerol and inositol (system 3, revealing agent 0.1 N $AgNO_3$–7 N NH_4OH, 1:1, v/v); from the PCs, choline and glycerol (system 2, Dragendorff reagent); from the PEs, ethanolamine and glycerol (system 2, ninhydrin); from the PGs, glycerol (system 2, 0.1 N $AgNO_3$–5 N NH_4OH–2 N NaOH, 1:1:2, v/v/v).

For the enzymolysis of the PCs, PGs, and PIs we used phospholipase A_2 from kufi venom [19]. The enzymolysis of the PCs was carried out by adding a solution of 1 mg of kufi venom in 0.4 ml of Tris buffer (pH 10.1) to a solution of 30 mg of the PC sample in 10 ml of diethyl ether. The mixture was stirred with a magnetic stirrer at room temperature for 1 h. After the end of enzymolysis the solvent was evaporated off to dryness under vacuum, and the residue was dissolved in chloroform–methanol (2:1, v/v). The hydrolysis products were separated by preparative TLC in system 1. The enzymatic hydrolysis of the PEs and the PGs was carried out similarly, with a time of 10 h for the PEs and 1 h for the PGs (pH 9.2).

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