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PHOSPHOLIPID COMPOSITION OF FUNGI MUTUALISTIC WITH *XYLEBORUS FERRUGINEUS*

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Abstract—TLC analyses revealed a qualitative similarity in the phospholipid compositions of three fungi, *Fusarium solani*, *Cephalosporium* spp. and *Graphium* spp., mutualistic with the ambrosia beetle, *Xyleborus ferrugineus*. Major components were phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI). PC and PE totalled more than 60% of the phospholipids in *Cephalosporium* spp. and *F. solani*, and more than 80% in *Graphium* spp. The higher combined percentage in *Graphium* spp. was due to PE content. Phosphatidylserine was not detected in the mutualistic fungi. In terms of lipid phosphorus per unit of dry weight, there was a 2:3:4 ratio for *F. solani* : *Cephalosporium* spp. : *Graphium* spp.

INTRODUCTION

EARLIER work established that three species of ambrosial fungi, *Fusarium solani*, *Cephalosporium* spp. and *Graphium* spp., were mutualistically involved in the nutrition of the beetle, *Xyleborus ferrugineus*.[1-3] Subsequent studies revealed unusual sterol requirements by this beetle when freed of its fungal ectosymbionts.[4,5] Because of the obligatory ectosymbiotic relationship between the beetle and its fungi, and of the general importance of lipids to insect development, a study of the phospholipid contents of the three fungi was undertaken. In this paper, the qualitative and quantitative phospholipid composition of these three fungal species are presented.

RESULTS

Total lipids from each of the three fungal species were extracted in $CHCl_3$–MeOH (2:1) and further separated into neutral and polar fractions. The polar lipids (2·8%, 3·6% and 4·5% of the total lipids, respectively, for *F. solani*, *Cephalosporium* spp. and *Graphium* spp.) were analyzed for phospholipid composition in one- and two-dimensional TLC using several adsorbants and different solvent systems. The phospholipids of the three fungi, identified by comparison of R_f values and staining reactions against authentic standards, are presented in Tables 1–3 for *F. solani*, *Cephalosporum* spp. and *Graphium* spp., respectively. Phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) were the major phospholipids in all three species. Cardiolipin, not detected in *F. solani* and *Cephalosporium* spp., was present in small amounts in *Graphium* spp. Several glycolipids, not visible with iodine vapor, appeared on charring with 50% H_2SO_4. One was detected in *Cephalosporium* spp. and three in the other two species.

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TABLE 1. TWO-DIMENSIONAL TLC OF THE POLAR LIPIDS OF *Fusarium solani* SHOWING R_f VALUES IN TWO SOLVENT SYSTEMS ON EACH ADSORBENT, AND STAINING REACTIONS

	R_f values*				Staining reactions†					Identification
	SGG(carbonate)‡		SGG(borate)‡							
Spot No.	1§	2	3	4	I_2	H_2SO_4	Nin	Bi	Ag	
0	0·0	0·0	0·0	0·0	+	+	−	−	−	Lipid remaining at origin
1	0·0	0·46	—	—	+	+	−	−	−	Unknown
2	0·09	0·69	0·33	0·28	+	+	−	+	−	Phosphatidylcholine
3	0·33	0·73	0·46	0·70	+	+	+	−	−	Phosphatidylethanolamine
4	0·16	0·52	0·22	0·71	+	+	−	−	+	Phosphatidylinositol
5	0·31	0·65	—	—	−	+	−	−	−	Glycolipid
6	0·66	0·66	—	—	−	+	−	−	−	Glycolipid
7	0·88	0·73	—	—	−	+	−	−	−	Unknown‖ close to cardiolipin
8	0·74	0·85	—	—	−	+	−	−	−	Glycolipid
9	—	—	—	—						Solvent front artifact
10	—	—	0·31	0·69	−	+	−	−	−	Unknown‖
11	—	—	0·31	0·66	−	+	−	−	−	Unknown‖

* Mean of 3 replications; − = no response.

† I_2 = iodine vapor; H_2SO_4 = 50% sulphuric acid; Nin = 0·2% ninhydrin ; Bi = bismuth (Dragendorff reagent); and Ag = ammoniacal silver nitrate.

‡ Adsorbant: SGG = Silica Gel G (Merck); SGG (carbonate) = Silica Gel G made up with 0·01 M sodium carbonate; and SGG(borate) = Silica Gel G made up with borate buffer at pH 8.

§ Solvent systems: 1 = $CHCl_3$–MeOH–AcOH–H_2O (250:74:19:3); 2 = $CHCl_3$–MeOH–7 N NH_4OH (230:90:15); 3 = $CHCl_3$–MeOH–7 N NH_4OH (60:35:5); 4 = $CHCl_3$–MeOH–7 N NH_4OH (35:60:5).

‖ Trace amounts.

\+ = Positive reaction.

TABLE 2. TWO-DIMENSIONAL TLC OF THE POLAR LIPIDS OF *Cephalosporium* spp. SHOWING R_f VALUES IN TWO SOLVENT SYSTEMS ON EACH ADSORBENT, AND STAINING REACTIONS

	R_f values*				Staining reactions†					Identification
	SGG(carbonate)‡		SGG(borate)‡							
Spot No.	1§	2	3	4	I_2	H_2SO_4	Nin	Bi	Ag	
0	0·0	0·0	0·0	0·0	+	+	−	−	−	Lipid remaining at origin
1	0·0	0·46	—	—	+	+	−	−	−	Unknown
2	0·10	0·70	0·33	0·27	+	+	−	+	−	Phosphatidylcholine
3	0·34	0·71	0·47	0·68	+	+	+	−	−	Phosphatidylethanolamine
4	0·17	0·50	0·22	0·71	+	+	−	−	+	Phosphatidylinositol
5	0·66	0·66	—	—	−	+	−	−	−	Glycolipid
6	0·93	0·73	—	—	−	+	−	−	−	Unknown‖
7	—	—	—	—						Solvent front artifact
8	—	—	0·32	0·67	−	+	−	−	−	Unknown‖
9	—	—	0·32	0·64	−	+	−	−	−	Unknown‖

For *, †, ‡, §, ‖ and + see Table 1.

TABLE 3. TWO-DIMENSIONAL TLC OF THE POLAR LIPIDS OF *Graphium* spp. SHOWING R_f VALUES IN TWO SOLVENT SYSTEMS ON EACH ABSORBENT, AND STAINING REACTIONS

	R_f values*				Staining reactions†					Identification
	SGG(carbonate)‡		SGG(borate)‡							
Spot No.	1§	2	3	4	I_2	H_2SO_4	Nin	Bi	Ag	
0	0·0	0·0	0·0	0·0	+	+	−	−	−	Lipid remaining at origin
1	0	0·47	—	—	+	+	−	−	−	Unknown
2	0·11	0·71	0·33	0·28	+	+	−	+	−	Phosphatidylcholine
3	0·35	0·73	0·46	0·68	+	+	+	−	−	Phosphatidylethanolamine
4	0·17	0·52	0·21	0·70	+	+	−	−	+	Phosphatidylinositol
5	0·39	0·60	—	—	−	+	−	−	−	Glycolipid
6	0·53	0·67	—	—	−	+	−	−	−	Glycolipid
7	0·74	0·66	—	—	−	+	−	−	−	Glycolipid
8	0·95	0·62	—	—	−	+	−	−	−	Possibly phosphatidic acid‖
9	0·93	0·77	—	—	+	+	−	−	−	Cardiolipin
10	—	—	—	—						Solvent front artifact
11	—	—	0·30	0·69	−	+	−	−	−	Unknown‖
12	—	—	0·30	0·66	−	+	−	−	−	Unknown‖

For *, †, ‡, §, ‖ and + see Table 1.

Quantitation of the different phospholipids in the three fungi is summarized in Table 4. Quantities of phospholipids in *F. solani* and *Cephalosporium* spp. were very similar, with a 1:1 ratio of PE:PC, and a smaller PI content. *Graphium* spp. had a much higher PE content; almost twice that of PC. In terms of phosphorus per unit dry weight, there was approximately a 2:3:4 ratio for *F. solani*: *Cephalosporium* spp.: *Graphium* spp. The high phosphorus content in the latter species reflected the high PE content.

TABLE 4. PHOSPHOLIPIDS* OF AMBROSIAL FUNGI CULTURED IN 100 ml NEUTRAL-DOX-YEAST MEDIUM† PER 500 ml FLASK, INCUBATED FOR 15 DAYS AT 28° AND 70% r.h. IN DARKNESS

	Species					
	Fusarium solani		*Cephalosporium* spp.		*Graphium* spp	
Phospholipid	μmol‡ (g dry wt)	Total phospholipid (%)	μmol‡ (g dry wt)	Total phospholipid (%)	μmol‡ (g dry wt)	Total phospholipid (%)
Phosphatidylethanolamine	7·01 ± 1·04	28·24 ± 4·20	11 33 ± 0 97	33·80 ± 2·88	25·88 ± 2 29	53·31 ± 4·73
Phosphatidylcholine	8·53 ± 0 95	34 34 ± 3 83	11·17 ± 0·55	33·32 ± 1 64	14·74 ± 1·57	30·36 ± 3·23
Phosphatidylinositol	3·59 ± 0 33	14 45 ± 1·34	1·70 ± 0·57	5 07 ± 1 71	2·78 ± 0·76	5·72 ± 1 56
Cardiolipin	0·0	0·0	0·0	0·0	1·18 ± 0 33	2 44 ± 0·69
Unknown	5·10 ± 0·49	20·53 ± 1·98	5·12 ± 0 77	15 28 ± 2·31	1 66 ± 0·35	3·43 ± 0·73
Phospholipid remaining at origin	0 61 ± 0·15	2·44 ± 0 62	4·20 ± 1·12	12 53 ± 3·33	2 30 ± 0 15	4 74 ± 0·32
Total	24·84 ± 3·0	100	33 52 ± 3·2	100	48 54 ± 6·0	100

* Mean of 4 replications ± S.E.
† See ref. 12.
‡ Phosphorus

DISCUSSION

The general qualitative phospholipid pattern was similar in the three studied ambrosial fungi. PC and PE together comprised more than 60% of the total phospholipids in *Cephalosporium* spp. and *F. solani*, and more than 80% in *Graphium* spp. The increased percentage in the latter was due to a higher PE content. The PE:PC ratio of 1:1 in the first two species was comparable to that of *Saccharomyces cerevisiae*[6] and *Lipomyces lipofer*.[7] The PE contents were in the upper portion of the 18–30% range previously reported in four other Moniliales, but PC contents were much lower than the 41–54% observed in these species.[8] In *Graphium* spp., PE predominated by about a 2:1 ratio over PC and was much higher than the PE content of previously studied fungi. The 14% of PI in *F. solani* was about three times that in *Cephalosporium* spp. and in *Graphium* spp., but was much less than the 22·4% in *Lipomyces lipofer*.[7] Although faint traces were detected in the cardiolipin region of the TLC from the other two fungi, cardiolipin could only be quantitatively determined in *Graphium* spp. This phosopholipid has also been demonstrated in several yeasts, *Candida*,[9] *Kloeckera*[10] and *Saccharomyces*,[11] but not in *Lipomyces*,[7] four Endomycetales or four Moniliales species.[10] A notable deviation from previously published results from fungi was the absence of a detectable concentration of phosphatidylserine in the three ambrosial fungi.

EXPERIMENTAL

Axenic culture of ambrosial fungi. The three ambrosial fungi, *Fusarium solani*, *Cephalosporium* spp. and *Graphium* spp., isolated from the mycangia (i.e. fungal repositories) of surface-sterilized adult beetles, were pure cultured on PDA medium and each was subsequently used as inoculum for axenic growth in chemically defined liquid Neutral-Dox-Yeast medium in 500 ml flasks.[12] Each fungus was grown for 15 days in darkness in an environmental chamber maintained at 28° and 70% R.H. At harvest, the fungal mats were washed in distilled water and then lyophilized.

Extraction procedure. Batches (15 g) of lyophilized fungus of each species were extracted for total lipids in $CHCl_3$–MeOH (2:1) by the methods of Folch *et al.*[13] The volumes of the lipid fractions were then reduced under vacuum and separated into neutral- and polar-lipid fractions by silicic acid (200 g) column chromatography. Two effluents were used to elute the total lipid fraction, first 500 ml $CHCl_3$ for neutral lipids and then 1 l. MeOH to collect the polar lipids. The MeOH fraction was dried under vacuum, weighed and then analyzed for phospholipids by TLC.

TLC chromatography. Polar lipid samples were applied to plates coated with 2·5 mm basic Silica Gel G and run, as described previously,[14–16] in one- and two-dimensional TLC. Solvents used in two-dimensional TLC were, by vol.: $CHCl_3$–MeOH–AcOH–H_2O (250:74:19:3) and $CHCl_3$–MeOH-7 N NH_4OH (230:90:15); and $CHCl_3$–MeOH–7 N NH_4OH (60:35:5) and $CHCl_3$–MeOH–7 N NH_4OH (35:60:5). The lipid spots were nonspecifically detected by iodine vapor and/or 50% H_2SO_4. Specific staining reagents used were ninhydrin for amino phosphatides, Dragendorff reagent for choline-containing lipids and ammoniacal $AgNO_3$ for inositol and glycerol. The fungal polar lipids were compared against authentic phospholipids, chromatographed singly and in mixtures (seven solvents, three adsorbants), for R_f values and staining

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reactions. All chromatograms were conducted in at least three replicates. For quantitative determinations three chromatograms were pooled as a replicate to ensure sufficient material for detection by color development of phosphates based on the methods of Kahovcová and Odavić.[17]

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Key Word Index—Fusarium solani; *Cephalosporium* spp.; *Graphium* spp.; mutualistic fungi; *Xyleborus ferrugineus*; ambrosia beetle; phospholipids.