Phytochemistry, 1978, Vol 17, pp 85–89 Pergamon Press Printed in England.

INFLUENCE OF GROWTH MEDIUM ON SURFACE AND WALL LIPID OF FUNGAL SPORES

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(*Received* 28 *June* 1977)

Key Word Index—*Alternaria tenuis*; *Botrytis fabae*; *Neurospora crassa*; *Rhizopus stolonifer*; fungi; spore surface; spore wall; growth medium; fatty acids; glycerides; alkanes.

Abstract—Conidiospores of *Alternaria tenuis*, *Botrytis fabae*, *Neurospora crassa* and sporangiospores of *Rhizopus stolonifer* from cultures on various media were shown by microelectrophoresis to have lipid on their surface. Analyses of lipid fractions obtained by sequential solvent extraction demonstrated that surface lipid forms a small but discrete layer different in composition from that within the wall. Free fatty acids, alkanes, triglycerides and other acyl lipids were identified by GC–MS. Phospholipids and sterols were absent. The qualitative and quantitative composition of both the surface and wall lipid fractions was dependent upon the growth medium used.

INTRODUCTION

Mycelial lipids of many fungi have been studied in detail [1, 2] but comparatively little is known about lipids in spores. Fatty acid components have been identified in uredospores of rusts [3, 4], conidiospores of *Fusarium* sp. [5] and *Penicillium* sp. [6] and sporangiospores of Phycomycetes [7]. Palmitic, stearic, oleic and linoleic acids were the major components but in conidiospores of *Erysiphe graminis* the C_{20}, C_{22} and C_{24} acids predominated [8]. Alkane components have been studied in rust spores [4, 9] where the C_{27}, C_{29} and C_{31} homologues predominate. Although ergosterol has often been considered to be the major sterol of fungi other sterols are sometimes more abundant in spores [10]. Spore phospholipids and triglycerides have also been identified [11, 12].

Few workers have distinguished spore wall lipids from intracellular fractions or examined the distribution of lipid in the wall. Bertaud *et al.* [13] identified fatty acids removed from the surface of *Pithomyces chartarum* spores and the composition of surface hydrocarbons from smut spores has been reported [14]. Attempts have also been made to correlate lipid extraction with changes in fine structure and surface morphology of smut and powdery mildew spores [8, 15].

Lipids in fungi can vary both quantitatively and qualitatively according to the growth conditions and medium [16, 17]. In an earlier paper [18] we used a microelectrophoretic technique to show that lipid is present on the surface of spores of some species but absent from others. The composition of lipid on the surface was compared with that within the wall. The present work, using improved analytical procedures, describes the effects of different growth media on the occurrence and the composition of surface and wall lipids.

RESULTS AND DISCUSSION

Alternaria tenuis, *Botrytis fabae*, *Neurospora crassa* and *Rhizopus stolonifer* were grown on various media selected for adequate spore production. Spores of all the fungi examined by microelectrophoresis showed a progressive increment in mobility with increasing concentrations of SDS (Table 1): this is indicative of a surface lipid layer [19]. Although surface lipid has previously been shown to be absent from some airborne fungal

Table 1. The effect of SDS on the electrophoretic mobility of some fungal spores suspended in Na–K phosphate buffer (pH 7; I = 0.01)

Organism	Medium	Electrophoretic mobility $10^{-8}m^2V^{-1}S^{-1}$	Percentage increase in negative mobility: Concentration of SDS (μM) 1	10	100
Alternaria tenuis	Carrot agar	−2.62	15	16	24
	Czapek-Dox	−0.96	12	29	31
Botrytis fabae	Peptone	−1.54	5	6	15
	Malt agar	−1.11	8	9	17
Neurospora crassa	Defined	−0.45	7	9	24
	Malt agar	−0.81	15	17	21
Rhizopus stolonifer	Malt agar	−1.78	3	5	33
	PDA	−1.96	9	12	22

spores [18], our results show that lipid is nevertheless present on spores of *A. tenuis*, *B. fabae*, *N. crassa* and *R. stolonifer* irrespective of the medium on which they are grown. Surface and wall lipids were separated from freeze-dried spores by a differential extraction procedure. The easy removal of lipids by extraction with hexane at room temperature confirmed that surface lipids were present, since this procedure will not extract lipid from within the wall or cytoplasm [18]. The presence of lipid on the spore surface is thus probably an inherent property of the species. The proportions of surface and wall lipid present were calculated by summation of the individual components assayed by GLC (Table 2). Although amounts of superficial lipid on the spores varied, only minute quantities were present. The amounts represented 500–8600 pg/cm^2 of surface area, though the lipid may not be evenly distributed. Although it is difficult to give an exact measure of the thickness of the surface layer implicated, the electrophoretic behaviour of the spores depended on the composition of about the outermost 3 nm of their wall. The gross compositions of lipid isolated from spore surfaces and spore walls are shown in Tables 3 and 4 respectively. The wide variations in the composition of the surface and wall fractions from the same species shows that the lipid composition of spores is affected by their chemical environment. Lipids in the media may be incorporated into the fungi or lipid precursors may influence fungal lipid biosynthesis.

Free fatty acid

Acids identified on the spore surfaces and within the walls were from C_{14} to C_{28}. Hexadecanoic acid always predominated, ranging from 79% of the surface acids on *B. fabae* spores from malt agar to only 44% of acids on *R. stolonifer* spores from carrot agar. A large proportion (14–46%) of octadecanoic acid was also present in the surface acid fractions. No free acids were detected on the surface of *B. fabae* spores from peptone agar medium, although esterified acids as the non-triglyceride acyl fraction comprised the main surface lipid component. In all other fractions, even carbon number acids predominated; small amounts of odd acids were also present, the C_{17} and C_{23} members being most abundant. The relative proportions of the free fatty acids on the spore surface were different from those in the corresponding walls, confirming the existence of a discrete lipid layer. Octadecenoic acid was the sole unsaturated acid

Table 2. The effect of growth medium on the amount of surface and wall lipid present on some fungal spores

Organism	Medium	Surface lipid μg/g spore dry wt	Wt 10^6 dry* spores (μg)	Surface area* 10^6 spores (cm^2)	Surface lipid pg/10^6 spores	Surface lipid pg/cm^2	Wall lipid μg/g dried wall
Alternaria tenuis	Carrot agar	22	180	5.11	3960	774	808
	Czapek-Dox	83	180	5.11	14940	2923	3119
Botrytis fabae	Peptone	50	130	5.06	6500	1284	1520
	Malt agar	63	130	5 06	8190	1618	373
Neurospora crassa	Defined	23	24	0.99	552	557	3343
	Malt agar	41	24	0.99	984	994	509
Rhizopus stolonifer	Malt agar	67	130	4.69	8710	1857	619
	PDA	311	130	4 69	40430	8620	12487

* From Richmond and Somers [37].

Table 3. The effect of growth medium on the composition of surface lipid on various fungi. Fractions expressed as % by weight of total surface lipid

	Alternaria tenuis		*Botrytis fabae*		*Neurospora crassa*		*Rhizopus stolonifer*	
Medium	Carrot agar	Czapek-Dox	Peptone	Malt agar	Defined	Malt agar	Malt agar	PDA
Alkanes	19.4	1.9	6 9	8.5	15.2	·	10.3	5.6
Triglycerides	26.8	11.0	—	9.6	3.8	–	3.5	25.1
Free fatty acids	43 0	40.8	—	29.8	42.9	40.0	52.3	35.0
Other acyl lipids	10.8	46.3	93.1	52.1	38 1	60.0	33.9	34 3

Table 4. The effect of growth medium on the composition of wall lipid of various fungi. Fractions expressed as % by weight of the total wall lipid

	Alternaria tenuis		*Botrytis fabae*		*Neurospora crassa*		*Rhizopus stolonifer*	
Medium	Carrot agar	Czapek-Dox	Peptone	Malt agar	Defined	Malt agar	Malt agar	PDA
Alkanes	1.8	0.8	0.9	7.0	4.4	—	1.6	—
Triglycerides	—	27.5	—	33.3	—	—	–	12.3
Free fatty acids	70.8	49.8	67.9	23.3	74.4	41.4	64.0	17 6
Other acyl lipids	27.4	21 9	31.2	36 4	21.2	58.6	34 4	70 1

detected in either surface or wall fractions; polyunsaturated acids, widely identified in total lipid extracts [1, 6] and 9,10 epoxyoctadecanoic acid, a major component of rust spore lipids [4, 10], were not detected. Free or esterified polyunsaturated acids have been identified in somatic lipids of all fungi so far analysed [20]; this absence from both surface and wall extracts provides additional evidence that cytoplasmic components were not extracted.

Triglycerides

Triglyceride esters of fatty acids were contained in some of the surface and wall extracts; their composition was medium dependent. Thus dodecanoic acid was the major triglyceride fatty acid (56%) on the surface of *A. tenuis* spores from carrot agar but was almost absent from the surface of spores on Czapek-Dox medium. Dodecanoic was also a major acid in surface triglycerides on *R. stolonifer* grown on malt agar (13%) or potato dextrose agar (18%) although the free acid was again absent. On other spores where surface triglycerides were present, hexadecanoic acid was usually the principal esterified acid ranging from 43–73% of the total; tetradecanoic and octadecanoic were also identified, but unsaturated acids were virtually absent. Only small amounts of odd carbon-number acids were esterified; the C_{15} member predominated. Triglycerides were also major components in the spore walls of *A. tenuis* grown on Czapek-Dox medium and *B. fabae* grown on malt agar. Tetradecanoic, hexadecanoic and octadecanoic acids were the major triglyceride acids; esters of decanoic, dodecanoic and octadecanoic acids were also present. Triglycerides occur widely in mycelium [9, 21–23] but have rarely been previously reported in spores. Bianchi and Turian [24] found less than 1% triglycerides in lipids from *N. crassa* spores, although they form a much larger proportion in mycelial lipids [25]. Triglycerides were not detected within spore walls of this organism, although small amounts were present on the surface of spores from the defined medium. Bianchi and Turian [24] found a considerable decrease in total spore triglycerides during germination and Marouf and Malhotra [26] have suggested that they form the immediate source of unsaturated acids in Phycomyces spores during germination and differentiation. We could find no correlation between free and triglyceride acids either on spore surfaces or within walls and it is evident that triglycerides did not serve here as precursors for free fatty acids.

Other acyl lipids

Non-triglyceride acyl lipids, mainly mono- and di-glycerides were present on spore surfaces and walls; esters of even carbon-number acids predominated. Hexadecanoic and octadecanoic acids were the principal acids identified but some surface fractions contained a considerable proportion of esters of decanoic and dodecanoic acid, although the corresponding free acids were not present. Some odd carbon-number acids were also identified; 9% of the total esterified non-triglyceride acid present in surface lipid on *R stolonifer* spores from malt-agar was pentadecanoic and 8.3% on *A. tenuis* spores from carrot agar was heptadecanoic. Only traces of unsaturated acids were detected. Non-triglyceride acyl compounds formed a large proportion of the lipid in all the spore walls. Although even carbon-number acids were also dominant within the walls, a considerable proportion (23%) of esterified pentadecanoic acid was found in spore walls of *R. stolonifer*. As with the surface fractions, esters of unsaturated acids were virtually absent.

Mono- and di-glycerides have been identified in mycelium of various fungi [21, 27, 28, 33] but have not previously been reported in spores. They formed the major part of the superficial lipid on some of the spores examined and were present even where triglycerides were absent; mono- and di-glycerides were evidently synthesized preferentially. Free fatty acids obtained by hydrolyses of non-triglyceride acyl lipid fractions corresponded with those from triglycerides; a common pool of fatty acids is probably involved.

Alkanes

Alkanes were present on the surface of all the spores except those of *N. crassa* grown on malt agar. They comprised the smallest fraction identified in most extracts and formed a lesser proportion of the wall lipid than that of the surface lipid. They were absent from the walls of some spores. Alkanes identified ranged from C_{18} to C_{36}, and the principal homologues were between C_{24} and C_{31}. Distribution patterns of alkanes in superficial lipid and in spore walls varied for different fungi and between spores of the same fungus grown on different media. In both fractions, there was an approximately equal distribution of alkanes of the odd and even series; a similar situation has been found in total lipids from *Penicillium* sp., *Aspergillus* sp., *Trichoderma harzianum* and yeasts [28, 29]. A predominance of odd series alkanes has been reported from the surfaces of smut spores [14, 15, 30] and rust spores [2, 31, 32], but the possibility that some of the lipid may have originated directly from the host plant cannot be ruled out; no odd carbon preference was found in alkanes from aeciospores of the rust *Cronartium fusiforme* [4]. Our results support the view that, unlike higher plants, fungi in common with other primitive organisms, contain approximately equal amounts of odd- and even-series alkanes. There was no correlation between fatty acids present and alkanes and it is unlikely that hydrocarbons are formed by direct decarboxylation of fatty acids; there is a similar lack of relationship in algae and bacteria [33]. Branched chain compounds and alkenes were absent from all the surface and wall fractions.

Wettability

A protective water-repellent function has sometimes been attributed to surface lipid on spores [13, 34]. Wettability of a substance is governed both by its chemical nature and its roughness [35]. All the spores studied were water-repellent but the wide qualitative and quantitative differences in surface lipid composition makes it unlikely that any single class of substituent is responsible. Spores from which surface lipids had been removed were still difficult to wet and lipids have been shown to be absent from the surface of other hydrophobic fungal and actinomycete spores [18, 36]. Although lipids may play some part in water-repellency, the physical conformation of the surface is probably more important.

EXPERIMENTAL

Organisms. Neurospora crassa Shear and Dodge (wild type 5297A) was obtained from the Commonwealth Mycological Institute, Kew; *Rhizopus stolonifer* (Ehrenb. ex Fr.) Lind from Botany Department, University of Bristol; *Botrytis fabae* Sardiña from Jealott's Hill Research Station, and *Alternaria tenuis* Nees from naturally infected material.

Media composition. N. crassa was grown on the defined medium of ref. [37] or on 3% malt agar. *R. stolonifer* was cultured on malt agar or on PDA, *B. fabae* on peptone medium [38] or on malt agar and *A. tenuis* on carrot agar (100 g mashed carrot/l.) or on Czapek-Dox agar.

Isolation of spore walls. Spores were harvested from cultures in petri dishes after 7 days growth at 25° [37]. After freeze drying, spore walls were obtained from spores from which surface lipid had already been removed (see extraction procedures). Dense spore suspensions were broken by shaking with an equal vol. of glass beads (Ballotini No. 12) in a Mickle disintegrator at 4° for 15 min. The spore walls were freed from cytoplasmic contamination by washing ×10 with 10% sucrose, ×5 with 0.9% NaCl and ×5 with H_2O [39] and finally dried under vacuum at room temp.

Wettability. Surface wettability was qualitatively assessed by completely covering the surface of a petri dish with spores and then gently adding one drop of H_2O. A non-spreading drop which remained intact and free to 'roll' was deemed not to wet the spore surface.

Electrophoretic measurements Spore mobilities were measured at 25° using a Zeiss Cytopherometer with platinum electrodes. Spores were suspended in Na–K phosphate buffer (pH 7, I = 0.01) or in buffer containing 1 μM, 10 μM or 100 μM SDS [40]. Each mobility was the mean of at least 20 observations; the standard error was less than 4% of the mean. The conductivity of buffered suspensions was measured with a Wayne Kerr B224 bridge.

Extraction and separation of lipids. Extraction procedures were based on those of ref [18]. Surface lipids were removed from freeze-dried spores (e.g. 2 g) by suspension in *n*-hexane (50 ml) for 1 min at room temp. After centrifuging, the supernatant fraction was decanted and filtered through close packed Et_2O extracted cotton wool to remove any residual spores. Wall lipids were extracted with 50 ml $CHCl_3$–MeOH (2:1) for 18 hr in a Bolton extractor. The solvents were removed using a rotary evaporator at 40°. Preliminary examination of spore extracts was carried out by TLC on Si gel G using (i) C_6H_6 for non-polar components (ii) *n*-hexane–Et_2O–HOAc (70:30:1) for more polar components and (iii) $CHCl_3$–MeOH–HOAc (85:15:8) for galactolipids and phospholipids. Components corresponding with alkanes, triglycerides, free fatty acids and a poorly resolved base-line fraction, tentatively identified as a mixture of mono- and di-glycerides, occurred in most of the extracts. Constituents were detected by spraying with H_2SO_4 and charring at 150° for 10 min No sterols (visualized by H_2SO_4 90°, 2 min), galactolipids (visualized by α-naphthol–H_2SO_4 100°, 5 min) or phospholipids (blue colouration with molybdenum reagent [41]) were found. PLC on 0.75 mm thick layers of Si gel G (20 × 20 cm plates) was used to isolate the various constitutents from the extracts after first methylating them with excess Et_2O–CH_2N_2 [42]. C_6H_6 was used for development and the separated bands were detected by briefly exposing to I_2 vapour; a typical fractionation gave the following R_f values: 0.85 (alkanes), 0.54 (fatty acid Me esters), 0.36 (triglycerides) and 0.05–0 (base-line). After evaporation of the I_2, bands were removed from the plates and the various fractions recovered from the Si gel by elution with Et_2O (50 ml). Recovered fractions were checked for purity by TLC in C_6H_6. Alkanes and fatty acid Me esters were identified by GLC comparison with authentic compounds and GC–MS. The amounts of each in extracts were also determined by GLC using response factors relative to *n*-tetracosane, calibrations being made with known ratios of pure compounds of comparable chain length. Triglyceride fractions were verified by IR (thin film) MS (probe) [43] comparison with glyceryl tripalmitate, and the amounts in extracts determined by GLC from the fatty acid Me esters [44] obtained by transesterification (3 hr) with 0.25 N NaOMe in MeOH–C_6H_6 (1 1). The base-line fractions from PLC showed strong IR carbonyl absorption 1740 cm^{-1}) and their MS typical acyl fragments [43]. These fractions were consequently designated non-triglyceride acyl lipids and assayed by GLC after transesterification using the response factors of ref. [44]. GLC was carried out using a FID detector and a 1 m × 2 mm i.d column packed with 1% Dexsil 300 on Supelcoport, N_2 flow rate 35 ml/min, programmed from 120° to 320° at 6°/min (injector 250°, detector 300°). For GC–MS the same Dexsil column was fitted to an LKB 9000 instrument operating at 70 eV with a trap current of 60 μA. The separator and ion source were both maintained at 270°. He was used as carrier gas (30 ml min) with temp. programming from 150° to 270° at 6°/min (injector 250°).

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