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SHORT COMMUNICATION

LIPID CONTENT OF *VERTICILLIUM ALBO-ATRUM**

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Abstract—Light-refractile granules in spores and mycelial cells of *Verticillium albo-atrum* R. & B. readily stained with Sudan Black B, indicating a marked lipid content. Mycelia contained 8% $CHCl_3$-methanol extractable material, whereas the spore content was 14 per cent (dry weight basis). GLC analysis showed that the principal fatty acids were palmitic, stearic, and oleic. Increased culture age did not result in any detectable cellular lipid changes. Spores starved in phosphate buffer progressively declined in lipid content.

INTRODUCTION

FUNGAL lipids are of considerable interest, mainly because of the high accumulation which is evident in some cases.[1] The metabolic significance of such accumulation evidently lies in their utilization as endogenous food reserves, as indicated by respiratory quotients of fungi deprived of exogenous substrate.[2–6]

Respiratory data for *Verticillium albo-atrum* R. & B. have shown similar results.[7] No study of the lipids of this important plant pathogen has been reported, but a conspicuous fatty component in cell-free preparations indicates a significant lipid content.[8,9] This investigation of lipid extracts from *V. albo-atrum* provides some data to substantiate these observations.

RESULTS AND DISCUSSION

Prominent refractile intracellular inclusions were microscopically evident in cells of *V. albo-atrum*. These granules readily stained with Sudan Black B (0·3% in EtOH), indicating them to be sites of lipid deposition. Generally 2–5 large granules per cell were present until an apparent transition to greater numbers of smaller size at an advanced cell age. This corresponds to the pattern of lipid deposition reported for *Oospora lactis*.[10] 'Wet' cells extracted with $CHCl_3$–MeOH and then examined by phase contrast microscopy still contained intact granules, but with evident reduced refractile quality. As expected, some lipid staining was evident also in cell walls and in the cytoplasm other than the above inclusions.

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Lyophilized spores of *V. albo-atrum* contained an average of 14% $CHCl_3$-methanol extractable material. TLC separation of the extract provided tentative identification of the major lipid classes, i.e. triglycerides, free fatty acids, phospholipids, and sterols. Spots were located by routine methods, and identification was based on R_f comparisons with lecithin, tristearin, palmitic and oleic acid, and cholesterol. A specific test for ergosterol[11] gave a positive reaction. Other unidentified lipid components were present. The preliminary findings show a probable lipid pattern consistent with that expected of higher fungi.[12]

The total fatty acid composition of the saponified lipid extract is shown in Table 1. The major constituents were palmitic, stearic, oleic and linoleic acids, comprising about 32, 35, 21 and 7%, respectively, of the total. In addition to those shown in the table, there

TABLE 1. TOTAL FATTY ACID DISTRIBUTION OF LIPID EXTRACTS FROM SPORES OF *V. albo-atrum* AFTER 10 days GROWTH

Fatty acid carbon chain*	Per cent distribution
Less than 14	2·1
14:0	0·6
15:0	0·3
16:0	31·7
16:1	0·4
17:0	0·4
17:1	0·2
18:0	35·1
18:1	21·0
18:2	6·5
18:3	0·4
18·4 (?)	0·4
20:1	0·9

* Number of carbon atoms in acid: Number of double bonds.

were traces of unidentified acids, apparently saturated and unsaturated branched components of C_{10}–C_{17} length. Comparing these results to those for other fungi,[12,13,14] palmitic and stearic acid contents are rather high. Oleic and linoleic acid contents compare with some fungi but not with others.

Extracts from cells of 20-day-old culture were made to determine whether culture age affected lipid content. No greater accumulation of intracellular lipids was evident; only 15% was extracted as compared to 14% for the 10-day period. Limited TLC examination revealed no marked changes in the lipid pattern from that obtained with younger culture cells. Further, GLC analysis of the saponifiable fraction showed essentially the same fatty acid pattern, with perhaps a slight shift to a more saturated state with ageing. Such a change appears to be characteristic of other fungi which have been examined.[12]

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Extracts were made from cells starved in phosphate buffer (0·1 M; pH 6) for increasing times in order to assess the utilization of lipids as endogenous substrates. Prior work indicated a shift to RQ's consistent with lipid oxidation when cells were deprived of exogenous substrate.[7] Cells from 10-day cultures were placed in the starvation medium and sampled daily for 16 days. Extractable lipid content decreased progressively from about 15% of dry wt. at the beginning of starvation to about 9% at 16 days, indicating a depletion of endogenous reserves and the importance of lipids as nutritional reserves in this organism. TLC observations showed an apparent preferential utilization of free fatty acids in this case, but the results are not unequivocal. While spore germination was not examined in relation to this depletion of lipid reserves, the extended starvation period and the consistent decline in lipid content would argue against this being the major factor in changing lipid content.

The mycelial lipid content was determined also. Mycelial growth from 10-day-old cultures was collected on cheesecloth and washed extensively to remove spores. After lyophilization, lipids were extracted and weighed. The total extractable lipid content averaged about 8%, as compared with 14% obtained from spores.

The data show that *V. albo-atrum* does not possess any particularly unique lipid characteristics as compared to some other fungi. Lipids do occur in significantly high proportions in this organism, and cytoplasmic refractile granules appear to be the primary sites of lipid deposition. The apparent dynamic condition of these granules, the utilization of lipid components as endogenous reserves, and the differences in lipid content of morphological forms are evidence of the importance of lipid metabolism to the physiology of this organism. Assessment of the importance of lipid metabolism in relation to parasitism of the host plant remains to be investigated.

EXPERIMENTAL

Verticillium albo-atrum, originally isolated from a diseased cotton plant, was grown in liquid culture on a platform shaker at about 100 c/min. The growth medium, Czapek Dox Broth, was seeded with plugs from stock potato-carrot-dextrose-agar cultures. Growth was at 25° in constant fluorescent light for either 10 or 20 days, depending upon the experiment. Spores (unicellular structures produced abundantly in liquid cultures) at harvest were separated from mycelia by filtration through four layers of cheesecloth. They were subsequently recovered by centrifugation, resuspended in wash water, and recentrifuged. Those not processed immediately were stored at −20° in N_2 until extraction. For starvation studies, 10-day-harvest cells were resuspended in 0·1 M pH 6 phosphate buffer at a volume equivalent to the original growth medium, placed on the shaker, and reharvested at specified times.

Cells were spread on a glass plate and lyophilized in a chamber containing $CaSO_4$ and surrounded by dry ice in an insulated box. Dried cells were then scraped from the plate and pulverized in N_2 in a ball mill at 5° for 12 hr. Microscopic examination showed virtually complete cell disruption. The resulting powder was weighed and extracted by shaking with $CHCl_3$–MeOH (2:1, v/v, 20 ml/g tissue) for 4 hr. The extract was filtered through defatted filter paper, and the residue and paper were thoroughly washed with the Folch solvent. The total filtrate was then washed with water (20% of the total vol.) according to the Folch method.[15]

The crude extract was examined by TLC, using 250 μm-thick Silica Gel_{254} films on 20 × 20 cm plates.[16] Plates were developed ascendingly for 100 mm, using light petroleum–Et_2O–HOAc (85:15:1, by vol.). Spots were located with UV, iodine vapor, H_2SO_4 and heat, Rhodamine 6G and a specific stain[17] for phospholipid. Further class identification was achieved using reference compounds. R_f comparisons were made with established data for complex lipid mixtures.[18]

Total fatty acid analysis was made on the fraction resulting from saponification, by routine procedure

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as outlined by Hammarstrand,[19] of the crude extract. After derivatizing with BF_3-methanol,[20] fatty acid methyl esters were extracted with petroleum for GC analysis.

GC analysis of fatty acids basically followed the procedure outlined by Hammarstrand.[19] The instrument used was a Varian Aerograph, Series 1200, Hi-Fi III Chromatograph, using either a polar (diethyleneglycolsuccinate (DEGS) 20% on 60/80 mesh chromosorb *W*) or a non-polar (Apiezon L, 10% on 50/60 mesh anakrom ABS) column, each 1/8 in. by 5 ft. Methyl esters in petroleum were injected in 1 μl charges, and temperature was programmed at 15°/min from 100 to 215°. The carrier gas was N_2 at a flow rate of 20 ml/min. Identification was made from retention time, using a plot of log relative retention time versus carbon number of standard acids.[21,22] Further identification with regard to unsaturation was made by comparison of retention times on DEGS and Apiezon L columns. Determination of fatty acid quantities was made from peak areas.[23]

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