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EXPERIMENTAL ARTICLES

Sporangiospores of the Fungus *Mucor lusitanicus* 12M: Correlation between Lipid Composition, Viability, and Morphology of Growth upon Germination

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Abstract—We studied viability of sporangiospores from a surface culture of the fungus *Mucor lusitanicus* 12M grown on wheat bran. With culture aging, the sporangiospores exhibited a tendency toward dimorphic growth upon germination and then lost the ability to germinate. This correlated with changes in the sporangiospore lipid composition, which involved a reduction in the total lipid pool and in the levels of reserve lipids and phospholipids in particular. We suggest that lipid catabolism in sporangiospores causes their defectiveness in the senescent culture.

Sporangiospores are specialized cells produced by mycelial fungi during their ontogenesis; by means of sporangiospores, asexual reproduction takes place [1]. Mucoraceous fungi produce sporangiospores only in surface culture. Desiccated sporangiospores can be stored for a long time. In the presence of water and a nutrient source, sporangiospores germinate, developing mycelium. Under stress conditions (anaerobic atmosphere, high glucose concentration, low pH) some species of the mucoraceous fungi develop yeast-like budding cells [2]. We have earlier reported that *M. lusitanicus* exhibits dimorphic growth upon germination of sporangiospores under stress conditions [3, 4]. We found that the lipid composition of yeast-like cells differs from that of mycelium cells. However, little is known about the relationship between the properties of sporangiospores and the fungus ontogeny. We have reported that *M. hiemalis* develops arthrospores upon inoculation of sporangiospores from senescent culture in fresh medium [5]. The duration of shelf life and viability of spores from the soil fungus *Trichoderma harzianum* were demonstrated to be affected by culture conditions: pH, the C/N ratio, and culture age [6]. The spores differed in their ultrastructure and the fatty-acid composition of their phospholipids. It was found for many fungal species that lipid metabolism plays an important role in the process of spore germination [7]. In this connection, we decided to investigate the relationship between sporangiospore properties and morphological processes occurring upon germination.

The aims of the present work were to study lipid composition and viability of sporangiospores obtained in various periods of cultivation of the fungus *M. lusitanicus* on wheat bran, as well as cell morphology upon spore germination.

MATERIALS AND METHODS

Mucor lusitanicus 12M [8] from the collection of the Institute of Microbiology was used throughout this study. Sporangiospores were obtained from the culture grown on wheat bran. Wheat bran (15 g) moistened with 20 ml of distilled water was placed in a 750-ml flask and autoclaved at 1 atm for 40 min. A suspension of a 7-day-old culture of *M. lusitanicus* grown on wort agar was used as the inoculum. Cultivation was performed at 27°C. Sporangiospores were washed off with sterile water and centrifuged. For determination of spore viability, 1 ml of the suspension containing 10^7 spores/ml was inoculated into 50 ml of fresh medium containing (g/l distilled water) glucose, 60.0; urea, 2.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; K_2HPO_4, 1.0; $FeSO_4 \cdot 7H_2O$, 0.01; and yeast extract, 0.5 (pH 6.8). Incubation was performed in 250-ml Erlenmeyer flasks containing 50 ml of medium with shaking (180 rpm) at 27°C. The germination of sporangiospores and cell morphology were examined under a light microscope.

Lipids were extracted as described by Folch [9]. Spores were triturated with quartz sand for better extraction. For determination of the fatty-acid composition, lipids were subjected to acid methanolysis [10] and analyzed by gas–liquid chromatography on a model 3700 chromatograph with a flame-ionization detector (Russia). The column contained polyethylene glycol adipate (15%) on Chromosorb W (40–60 mesh), the temperature was 170°C, and the carrier gas (helium) flow rate was 40 ml/min.

Lipid fractions were separated by thin-layer chromatography using Kiesegel 60 F_{254} plates (Merck,

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Table 1. Fatty acids of total lipids of sporangiospores of *M. lusitanicus* 12M cultures of different ages

Day	Fatty acids, % of total							
	14 : 0	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	18 : 3	Δ/100 mol
6	2.00	30.28	1.75	5.02	18.30	28.00	14.50	119.55
14	2.40	30.10	1.81	6.49	25.77	19.00	14.66	115.56
25	2.75	29.65	0.34	3.79	21.72	23.79	17.93	123.43

Germany). Neutral lipids were developed with a mixture of hexane, diethyl ether, and acetic acid (80 : 20 : 1, vol/vol). Polar lipids were developed with a 60 : 25 : 5 (vol/vol) mixture of chloroform, methanol, and 28% ammonia, and a 6 : 8 : 2 : 2 : 1 (vol/vol) mixture of chloroform, acetone, methanol, acetic acid, and water. The spots were visualized by spraying the plate with either 10% phosphomolybdic acid in methanol or strong sulfuric acid with subsequent heating.

Lipid spots were identified by comparing their R_f values with those of authentic standards (Sigma, USA) and in qualitative reactions with Vas'kovskii reagent (phospholipids), ninhydrin (nitrogen-containing lipids with free amino groups), naphthol (glycolipids), Dragendorff reagent (choline-containing lipids), or a mixture of sulfuric and acetic acids (1 : 1, vol/vol) (free and esterified sterols).

For quantification of individual lipid classes, thin-layer chromatograms were scanned on a Mustek-ACEP scanner and analyzed with the use of the Rastr-Pro program.

The experiments were conducted in six replicates.

Analysis of variance, performed using Microsoft Excel-2000 software, used Student's criterion $P < 0.05$.

RESULTS AND DISCUSSION

We studied the morphology of cells developing from sporangiospores of the fungus *M. lusitanicus* 12M cultivated on wheat bran for 4, 6, 14, and 25 days. Sporangiospores from 4-day-old and 6-day-old cultures gave rise only to hyphal growth. However, germination of sporangiospores collected from a 14-day-old culture resulted in both hyphal and yeast-like growth. After sporangiospore swelling with the formation of spherical cells, some of the latter produced a germ tube and others developed buds. Most of the sporangiospores from the 25-day-old culture did not germinate. We observed under a microscope a quantity of spherical cells with a thick membrane that did not develop mycelium or buds and later degraded. However, supplementing culture broth with exogenous lipids (sunflower seed oil) initiated germination of spherical cells and further growth. In the presence of exogenous lipids, the yeast to hyphae transition was observed. Sporangiospores from older cultures did not germinate or transform into spherical cells even in the presence of exogenous lipids.

We earlier reported dimorphic growth of some mucoraceous fungi under aerobic conditions. In particular, this proved to be characteristic of *M. hiemalis* [11], which had been considered incapable of yeast-like growth. We suggested that dimorphism is characteristic of a wider range of mucoraceous fungi than was generally believed.

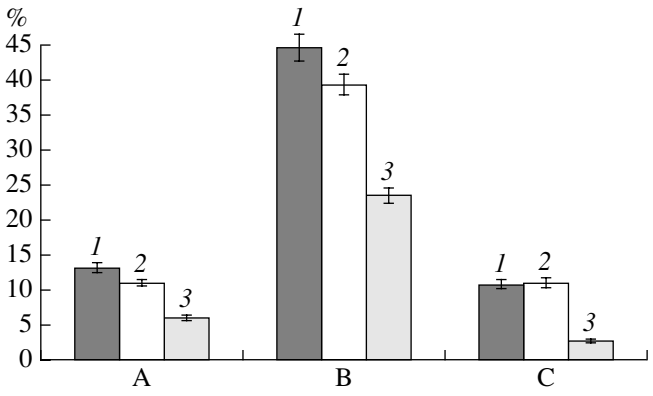
Our supposition is confirmed by the results of this study, which showed that morphogenetic processes during ontogenesis are strongly influenced by sporangiospore age. Extension of the time of cultivation of *M. lusitanicus* 12M on wheat bran resulted in gradual loss of spore viability. It should be noted that spores washed off after 6 days of cultivation and dried could be stored for 60 days at 20°C without any particular detriment to their viability. It was of interest to try to find out what processes occurring in sporangiospores resulted in the morphogenesic changes observed.

It is known that endogenous lipids play an important role in spore germination [12]. At early stages of germination, lipases are activated and lipid degradation is initiated. Freed fatty acids undergo [beta]-oxidation. The energy released is used in the processes of biosynthesis occurring during germination, including de novo synthesis of fatty acids and phospholipids incorporated into de novo synthesized membranes.

In this connection, we studied lipid and fatty acid composition in sporangiospores from spore-forming cultures of various ages. The most abundant fatty acids were palmitic and linoleic acids. It should be noted that the fatty acid composition did not vary greatly in sporangiospores of different age. However, sporangiospores from a 25-day-old culture contained larger quantities of γ-linolenic acid, and had a higher degree of fatty acid unsaturation than a 6-day-old culture (see Table 1).

The total lipid content in sporangiospores decreased with culture age from 13% of dry biomass weight (6-day-old culture) to 7% (25-day-old culture). We observed a decrease in triacylglycerols (TAG) and sterol esters (SE), which are considered to be reserve lipids, and in basic phospholipids (PL) (see figure). As a result, sporangiospores from a senescent culture contained larger quantities of free fatty acids (FFA), diacylglycerols (DAG), and free sterols in the fraction of neutral lipids (see Table 2).

The composition of polar lipids in sporangiospores also changed with culture ageing: there was a decrease in the level of basic membrane phospholipids (see Table 3). Sporangiospores from a 25-day-old culture



Changes in content of total lipids (A) and in proportions of reserve lipid fraction (B) and phospholipid fraction (C) in sporangiospores of the fungus *M. lusitanicus* 12M during cultivation. Fraction composition: (B) TAG + SE + FAE; (C) PC + PEA + PS. Culture age: (*1*) 6-day culture; (*2*) 14-day culture; (*3*) 25-day culture.

contained 3.5 times less phosphatidylcholine (PC), 9 times less phosphatidylserine (PS), and 1.5 times less phosphatidylethanolamine (PEA) than 6-day-old culture spores, whereas the PEA/PC ratio increased from 0.39 to 0.94. First stage germination was characterized by lipase activation and, in the first place, active degradation of PC. Phospholipid degradation resulted in an increase in the levels of phosphatidic acid (PA) and glycolipids in the polar lipid fraction. The PL to glycolipid (GL) ratio declined from 1.2 in young spores to 0.49 in senescent ones. Five glycolipids characterized by different R_f values were found. We observed an increase in the quantities of GL-1, having a free amino group, and GL-4, characterized by an R_f value close to that of cerebrosides. The suspension of spores from a 14-day-old culture was heterogeneous: dimorphic growth was observed upon germination. By this reason, we actually obtained lipid characteristics that are average for spores of different types. The decrease in TAG and fatty acid esters (FAE) paralleled the increase of DAG and free sterols in the neutral lipid fraction during cultivation. Phospholipid composition did not change significantly.

Spore suspensions from a 6-day-old culture and a 25-day-old culture were relatively homogeneous. All spores from the 6-day-old culture germinated, developing mycelium. The sporangiospores from the 25-day-old culture were nonviable. They exhibited significant differences in both total lipid content and phospholipid composition particularly.

Thus, catabolic processes in sporangiospores from senescent culture are similar to those in germinating spores [7, 12], but the processes of lipid synthesis do not proceed in them. Evidently, the degradation of reserve lipids in some of 14-day-old spores causes deficiency in the energy supply and results in the emergence of yeast-like cells. A connection between inhibition of energy-yielding processes and yeast-like growth has previously been suggested [13]. Dimorphism may also result from suppression of fatty acid synthesis, as in *M. racemosus* [14]. Involvement of membrane phospholipids in the processes of lipid catabolism is more pronounced in senescent spores. These phospholipids regulate intracellular processes as well as function as structural elements [15]. It is possible that a decrease in membrane phospholipids to a critical level results in the loss of spore viability.

Table 2. Lipid classes in sporangiospores of *M. lusitanicus* 12M cultures of different ages (% of total lipids)

Day	Polar lipids	Neutral lipids							
		DAG	desmethyl-sterols	methyl-sterols	FFA	quinones	TAG	FAE	SE
6	21.11	8.56	10.77	1.16	11.49	2.18	24.58	5.00	15.13
14	22.02	11.13	13.57	1.09	10.46	2.34	20.64	1.04	17.70
25	16.27	19.43	15.37	15.37	16.85	traces	16.42	–	7.21

Note: DAG, diacylglycerols; FFA, free fatty acids; TAG, triacylglycerols; FAE, fatty acid esters; SE, sterol esters.

Table 3. Polar lipid fractions in sporangiospores of *M. lusitanicus* 12M cultures of different ages (% of total)

Day	GL-1	PS	PA	GL-2	GL-3	PC	PEA	CL	GL-4	Unidentified PL	GL-5	PEA/PC	PL/GL
4	16.68	9.35	7.55	4.54	3.12	29.78	11.52	2.53	7.47	1.55	6.89	0.39	1.2
14	18.75	8.23	6.01	5.34	2.39	28.26	13.04	2.69	10.19	traces	5.32	0.32	2.2
25	33.33	1.07	12.09	5.91	4.43	8.14	7.68	3.94	18.22	traces	6.89	0.94	0.49

Note: GL, glycolipids (1, R_f = 0.04; 2, R_f = 0.24; 3, R_f = 0.28; 4, R_f = 0.6; 5, R_f = 0.7); PS, phosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PEA, phosphatidylethanolamine; CL, cardiolipin; PL, phospholipid.

It has been reported that mucoraceous fungi exhibit dimorphic growth under stress conditions [2]. No attention has been paid, however, to the properties of spores as the reason for dimorphism.

Our study of the growth of the fungus *Mucor lusitanicus* 12M on wheat bran showed that, with ageing of the spore-forming culture, sporangiospores exhibited a tendency toward dimorphic growth upon germination and then lost the ability to germinate. We found that, during culture ageing, processes of lipid catabolism occur in sporangiospores, resulting in a reduction in the level of reserve lipids and phospholipids. We suggest that changes in the lipid composition cause morphogenetic changes and a loss of germinating ability. This supposition is confirmed by the fact that supplementation of defective spores with exogenous lipids resulted in normal germination and further development. In *M. lusitanicus* 12M grown on wort agar, the decrease in the sporangiospore viability with the age of the culture is not as drastic, probably due to the nutritive value of the cultivation medium.

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