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LIPID CONTENT DURING SEXUAL DEVELOPMENT IN THE HOMOTHALLIC MUCOR *ZYGORHYNCHUS MOELLERI*

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Key Word Index—*Zygorhynchus moelleri*; Mucorales; triglycerides; sexual differentiation; trisporic acids.

Abstract—In *Zygorhynchus moelleri*, a homothallic Mucor, triglycerides are the main components of chloroform/methanol extractable lipids. The triglycerides accumulate in the aerial hyphae, particularly in the developing zygospores and in the lateral suspensors, but only after zygospore maturation. They are probably transported from the submerged mycelium to the aerial hyphae. Most of the fatty acid synthetase activity is found in the submerged mycelium. The fatty acid composition of the triglycerides does not change appreciably during sexual development. No influence of trisporic acids has been found on triglyceride synthesis or transport.

INTRODUCTION

Sexual reproduction in the Mucorales takes place by fusion of two gametangia which are derived from the thalli of two different mating types (heterothallism) or from one thallus (homothallism). In the homothallic *Zygorhynchus moelleri* one gametangium is produced terminally on a lateral aerial hypha after growing back and contacting the main hypha. The second gametangium is formed on the main hypha at the site of contact [1]. The two hyphae are different in several morphological and physiological respects. (1) The main hypha is delimited from the rest of the mycelium by a septum which is located just above the origin of the lateral hypha. (2) The lateral hypha shows a positive tropism towards the upper part of the main hypha. (3) The main hypha also attracts *plus* sexual hyphae from neighbouring heterothallic strains in interspecific copulations. Thus, the main hypha shows a *minus* tendency [2]. This idea is also supported by the fact that the cytoplasm of the main hypha contains a dehydrogenase involved in sex hormone (trisporic acid) production, that is characteristically found only in *minus* heterothallic species [3]. This suggests that the hormone system, found in heterothallic systems, is also operative in aerial parts of this homothallic species. (4) Another difference between the conjugating main and lateral hyphae is the presence of oil globules in the lateral [1]. This leads to the question of whether the accumulation of lipid is induced by trisporic acids or by their precursors, for trisporic acids are known to have a stimulatory effect on isoprenoid production in several mucoraceous fungi [4], and to change the ratio of classes of lipids in the *heterothallic Blakeslea trispora-minus* [5].

In this investigation we compared a sexually incompetent (agamic) strain with a competent strain to correlate sexual development with lipogenesis and to study the effect of trisporic acids.

RESULTS

Sexual behaviour of the agamic strain of Zygorhynchus moelleri

Zygospore formation in this strain can be induced by addition of sex hormones isolated from the heterothallic *Blakeslea trispora*, namely the trisporic acids, the 4-dihydromethyltrisporates and the trisporins. Maximal zygospore production by the induced agamic strain (on addition of 50μg trisporic acids or trisporins or 100μg 4-dihydromethyltrisporates in a well before the mycelial front) was however only 5% of that of the fertile strain under the same conditions. In contrast to the fertile strain, *Z. moelleri*-agamus did not produce any detectable hormones, though the fungus was able to convert 4-di-

Table 1. Lipid content of *Zygorhynchus moelleri* and the agamic strain

Lipids in	*Z. moelleri*	*Z. moelleri-agamus*
Phospholipids	6.5 (0.4)	6.8 (0.4)
Sterols	1.7 (0.2)	2.1 (0.1)
Sterolesters	t	t
Monoglycerides	t	t
Diglycerides	t	t
Triglycerides*	32.7 (1.0)	18.3 (2.1)

* Triglyceride content from three independent experiments (all in triplicate were for *Z. moelleri* 25.6 (6.6) and for *Z. moelleri*-agamus 13.2 (4.7).

Values are expressed as percentages of dry weight of the mycelium; t: trace. The values are averages of triplicates; between brackets standard deviations are indicated. The mycelia were grown on SM for three days. Lipid extraction, purification and determination were carried out as described in the Experimental.

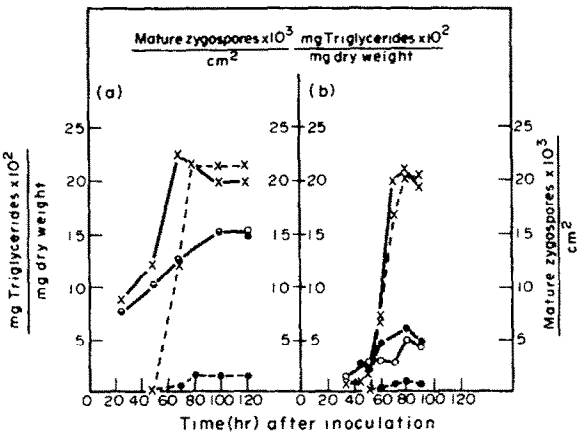

Fig. 1. Triglyceride content and zygospore production in *Zygorhynchus moelleri*. (a) Total mycelium: (b) aerial hyphae. At time zero the cultures were inoculated in SM. The extractions were performed as described in Experimental. Symbols with solid lines represent triglyceride contents and symbols with dotted lines represent zygospore production. Values are averages of triplicates; standard deviations are ⩽5% of the values indicated. ×—×: *Z. moelleri*; ●—●: *Z. moelleri*-agamus, treated with trisporic acids (1 mg/25 ml SM) at time zero; ○—○: *Z. moelleri*-agamus, untreated.

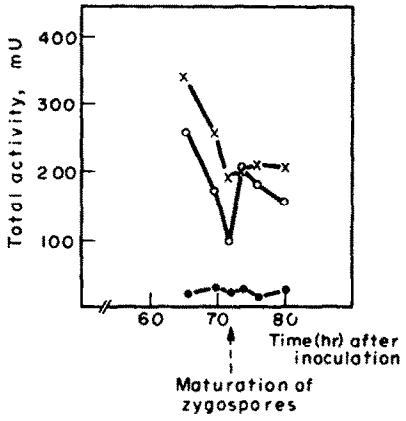

Fig. 2. Fatty acid synthetase activity in *Zygorhynchus moelleri*. Mycelia (of *ca* similar fr. wt) were frozen in liquid N_2, after which the aerial hyphae were separated from the submerged mycelium. After thawing at 0°, the fatty acid synthetase complex was isolated and the enzyme activity was determined as described in Experimental. The ordinate gives total activities of one mycelial mat; values are averages of triplicates; standard deviations are ⩽14% of the values indicated. ×—×: total mycelium; ○—○: submerged mycelium; ●—● aerial hyphae.

hydromethyltrisporates to trisporic acids. It is possible that its sterility is caused by the inability to produce hormones, although its low response to added hormones is another obvious limitation.

Triglyceride contents and fatty acid compositions during sexual development

Chloroform/methanol extracts of three-day-old mycelia contained triglycerides, sterols, phospholipids, traces of sterolesters and mono- and diglycerides. Table 1 shows that in both strains the triglycerides were the main components of extractable lipids. As is shown in Fig. 1a, the difference in triglyceride content between *Z. moelleri* and the agamic strain increased after zygospore formation in *Z. moelleri*. No difference in increase in triglycerides was found in the agamic strain and in the agamic strain treated with trisporic acids. Fig. 1b shows that the increase in triglycerides in *Z. moelleri* was most spectacular in the aerial hyphae. It nearly accounted for the increase found in the total mycelium. Also, a small though consistent difference was found between the triglyceride content of the aerial hyphae of the agamic strain and that of the trisporic acid-treated agamic strain. The difference was too small to be detected in extracts of total mycelia. Trisporic acid treatment 24 hr after inoculation did not alter the time of zygospore formation in *Z. moelleri* nor the number of zygospores formed. Neither could an effect on triglyceride content be found. During sexual development in *Z. moelleri* there was no change in the fatty acid composition of the triglycerides (Table 2). The fatty acid composition of the triglycerides in the agamic strain was similar to that of *Z. moelleri* and was not affected by trisporic acid treatment. So the action of trisporic acids seems to be restricted to the stimulation of zygospore formation in the agamic strain, where the

Table 2. Fatty acid composition of triglycerides in *Zygorhynchus moelleri* and the agamic strain during sexual development

	Z. moelleri				*Z. moelleri*-agamus with TA	*Z. moelleri*-agamus without TA
Hours after inoculation	40	56	71	78	78	78
Fatty acids						
14:0	1.8 (0.7)	2.7 (0.8)	3.9 (0.8)	2.3 (1.0)	1.6 (0.0)	1.4 (0.4)
16:0	0.6 (0.1)	0.6 (0.1)	—	—	—	—
18:0	31.1 (1.6)	32.5 (6.8)	38.7 (3.2)	32.4 (0.2)	32.5 (2.5)	32.7 (1.6)
18:1	12.8 (1.0)	17.3 (0.4)	17.7 (0.7)	10.7 (1.0)	10.3 (0.3)	8.2 (0.3)
18:2	46.3 (4.6)	41.6 (1.4)	27.5 (2.7)	47.2 (0.6)	44.1 (1.5)	43.4 (1.8)
18:3	3.1 (1.2)	2.0 (0.4)	3.4 (0.2)	5.3 (1.2)	7.5 (1.3)	5.6 (1.5)
20:0	4.3 (0.3)	4.0 (0.3)	6.5 (1.5)	2.1 (0.2)	4.0 (0.3)	4.4 (0.0)

Values are expressed as percentage of total; — absent. The values are averages of triplicates; standard deviations are indicated between brackets. TA (trisporic acids, 1 mg/25 ml SM) were added at the time of inoculation.

small increase in triglyceride content in the aerial hyphae can be correlated with the weak stimulation of zygospore formation. In *Z. moelleri* the number of zygospores formed on SM was less than on 30% beerwort, and also the amount of triglycerides in the aerial hyphae was less on SM than on beerwort.

Fatty acid synthetase activity in aerial hyphae and submerged mycelium in sexually developing Z. moelleri

Histochemically, the presence of lipids could be demonstrated in the submerged mycelium, and to a less degree in the sporangiophores and sporangiospores. However, on zygospore maturation both the lateral suspensors and spores themselves became loaded with lipids. This accumulation of lipids in the lateral suspensors might indicate another difference in metabolism between the two copulating hyphae. Therefore, fatty acid synthetase activity was measured in submerged and aerial hyphae. Fig. 2 shows that most of the activity was found in the submerged mycelium and only a small part was found in the aerial hyphae, even during the pronounced increase in triglyceride content of the aerial hyphae. If fatty acid synthetase activity was expressed per mg dry weight of the 37000 G pellet, which approximated the dry weight of the mycelial material, the specific activities for both submerged and aerial hyphae was 5 ± 1 nmol NADPH oxidized per min per mg dry weight. Therefore, the low activity in the aerial hyphae in itself suggests that transport of triglycerides from the submerged to the aerial hyphae occurs during zygospore maturation.

Triglyceride transport to aerial hyphae in sexually developing Z. moelleri

U-^{14}C-labelled glucose is readily incorporated into triglycerides in *Z. moelleri*. When labelled glucose was given just before the onset of zygospore maturation, it was immediately incorporated into the triglycerides of submerged mycelium and aerial hyphae; the specific activities of both fractions were the same (Fig. 3a and b). This suggests that triglycerides are transported from the submerged mycelium into the aerial hyphae. If triglyceride synthesis had taken place in the aerial hyphae one would have expected a significantly higher specific activity in the aerial hyphae compared with the submerged mycelium because the triglyceride pool in the aerial hyphae was negligable in comparison with the pool in the submerged mycelium at the time radioactivity was supplied. Another possibility, that triglyceride precursors were transported to the aerial hyphae and were incorporated there into the triglycerides when zygospore maturation started, was tested as follows. U-^{14}C-glucose was given 4 hr before the triglyceride accumulation in the aerial hyphae started. This led to an immediate incorporation of label in the submerged mycelium, while hardly any radioactivity was found in the aerial hyphae until zygospore maturation started (Fig. 3c). From then on, the specific radioactivity of the triglycerides accumulating in the aerial hyphae followed the specific activity of the triglycerides in the submerged mycelium (Fig. 3d). Though precursor transport induced at the moment of zygospore maturation cannot be excluded, the parallelism in specific activity of the triglycerides in submerged and aerial hyphae, together with the low fatty acid synthetase activity in the aerial hyphae, suggests that triglyceride accumulation in the aerial hyphae during zygospore maturation is mainly due to triglyceride transport from the submerged mycelium.

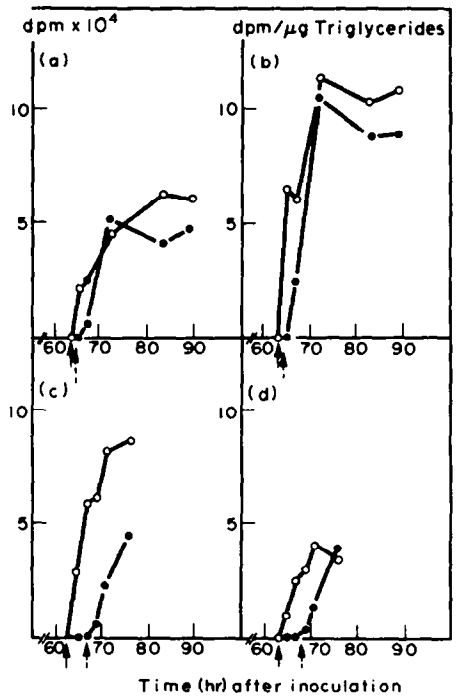

Fig. 3. Incorporation of radioactivity in aerial and submerged hyphae of *Zygorhynchus moelleri*. U-^{14}C-glucose (3 μCi) was added to the culture medium under the mycelial mat just prior to zygospore maturation (a and b) or four hours earlier (c and d). Aerial hyphae (●—●) were separated from the submerged mycelium (○—○), after which the materials were lyophilized and extracted. Triglycerides were purified and determined as described in Experimental. Values are averages of triplicates; standard deviations are ≤8% of the values indicated. a and c: Radioactivities in total extracts; b and d: radioactivities in triglycerides; ——→: U-^{14}C-glucose added; - - - →: start zygospore maturation.

DISCUSSION

It has been established that cultural conditions (age, temperature, carbon source) strongly determine the content and composition of lipids in fungi [6]. Safe [7] has shown in *Mucor rouxii* that the degree of unsaturation is dependent on the age of the culture and on the temperature. Deven and Manocha [8] compared fatty acid composition in *Choanephora cucurbitarum* under different conditions (age, temperature, pH and light) and showed that a decrease in the overall degree of unsaturation was almost entirely due to a decrease in α-linolenic acid content. We have studied the fatty acid composition in *Zygorhynchus moelleri* in conjunction with sexual development and have found that the lipid content considerably increases with time due to an increase in triglycerides, but without significant change in fatty acid composition. The sharp increase in triglyceride content in *Z. moelleri* can be correlated with zygospore formation; a similar, but small increase is found in *Z. moelleri*-

agamus, which produces only small numbers of zygospores when trisporic acids are added.

Trisporic acids, sex hormones in the heterothallic Mucors, are also thought to be involved in sexual development in homothallic Mucorales [3], but they only influenced the triglyceride content of aerial hyphae of *Z. moelleri*-agamus in as much as they stimulated zygospore formation. We found no influence of trisporic acids on fatty acid composition.

We have shown that the triglycerides which accumulate in the aerial hyphae of *Z. moelleri* during zygospore maturation, are probably synthesized in the submerged mycelium and transported to the aerial hyphae. This is in accordance with the work of Debell and Jack [9], who found that the positional (stereospecific) distribution of fatty acids in glycerolipids from mycelial and sporangial hyphae of *Phycomyces blakesleeanus* were similar. The explanation they suggested for this similarity was that identical lipids were synthesized in physically separate places or alternatively, that the biosynthesis took place in the mycelial mat and the lipids were transported to the sporangiophores. In our system, the low total synthetase activity in the aerial hyphae, and the results of our label experiments, make the second possibility more probable.

EXPERIMENTAL

Cultures and materials. The strains used were *Zygorhynchus moelleri* Vuill., CBS 501.66 and *Z. moelleri* race *agamus*, CBS 444.65, both obtained from the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. Trisporic acids to induce zygospores, were derived from *Blakeslea trispora* and isolated as described earlier [10].

Cultivation. The strains were cultivated at 20° in the dark in Petri dishes in 25 ml 30% (v/v) beer wort adjusted to pH 8 before sterilization, or in 25 ml synthetic medium (SM) containing 2% (w/v) glucose, 1% KNO_3, 0.5% KH_2PO_4, 0.25% $MgSO_4.7H_2O$, 0.1% yeast extract (Difco) with or without 1.5% agar.

Lipid extraction, separation and analysis. After removal of the culture medium, the mycelial mats were frozen in liquid N_2 and the aerial hyphae were scraped off the upper surface of the mats with a surgical knife. The mats, aerial hyphae or the total mycelia were lyophilized separately, and extracted in $CHCl_3$–MeOH (2:1) for 12 hr at room temp. with vigorous shaking. After filtration, the extracts were concd *in vacuo* and separated by TLC on Sil gel HF 254 with hexane–Et_2O–MeOH (18:6:1) followed by petrol [10]. The lipid bands were identified in UV and/or after spraying with H_2SO_4–EtOH (1:3) and heating for 5 min at 100°. They were scraped off and the lipid content was determined according to ref. [11]. As standards, phosphatidylcholine, cholesterol and glycerin tripalmitate were used. The radioactivity in each band was measured after mixing each sample with 10 ml xylene containing 25% Triton X-100, 0.03% POPOP and 0.3% PPO. The fatty acid composition of the triglyceride fractions was determined by GLC. Triglycerides were saponified in refluxing $CHCl_3$–MeOH–H_2SO_4 (100:100:1) for 90 min, after which methyl esters wre analyzed in a glass column (3.66 m × 3.27 mm) packed with 20% diethylene glycol succinate on Varaport W 60–80 mesh at 205°. As quantitative internal standard a known concn of methyl palmitate was added to each sample. Relative retention times and concns of fatty acid methyl esters were checked with palmitic, stearic, linoleic, linolenic and arachidic methyl esters as standards.

Fatty acid synthetase activity. Measured according to ref. [12]. The enzyme complex was isolated by a slightly modified method according to ref. [13]. The mycelia were homogenized at 0° in 0.2 M K phosphate buffer, pH 6.3, with 2 mM ethylenediaminetetraacetic acid disodium salt and 1 mM dithiothreitol, for 2 min in a Sorvall Omnimixer at full speed. The homogenate was sonicated (2 min, 8 microns) and then Triton X-100 was added (final concn 2%, v/v). After 15 min at 4°, the cell debris was removed by centrifugation for 20 min at 37000*g*. The supernatant was centrifuged for 90 min at 150000*g* and the pellet was used as enzyme prep. Enzyme activities were expressed as mU; 1 mU is the amount of enzyme which oxidizes 1 nmol NADPH per min. Protein concs were determined according to ref. [14].

Histochemical demonstration of lipids. Glutaraldehyde fixed mycelia (5 min, 5% glutaraldehyde) were washed with H_2O and stained with 0.2% Sudan III in EtOH–glycine 1:1, v/w). After 5 min the mycelia were washed with glycerol and examined under the microscope.

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