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EXPERIMENTAL ARTICLES

Lipid Composition of the Mycelium of the Fungus *Mucor hiemalis* Cultivated with Trehalose, Triacylglycerols, and Itraconazole

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Abstract—We investigated the growth and cell lipid composition of the fungus *Mucor hiemalis* VKMF-1431 cultivated under aerobic conditions in the presence of the morphogenetic agents itraconazole, exogenous triacylglycerols, and trehalose. The sporangiospores of a 6-day culture were used as inocula. Under these conditions, the fungus produced mycelium; nevertheless, solitary yeastlike cells also developed on the glucose-containing medium and in the presence of itraconazole and sterilized triacylglycerols (sTAGs). No yeastlike growth occurred in the system with trehalose and with unsterilized (native) TAGs (nTAGs). With trehalose and nTAGs in the cultivation medium, the ratio between PEA and PC, the two main types of membrane lipids, was low. This testified to a relatively high PC percentage and, accordingly, a stable structure and a highly functional state of the membranes. Moreover, if the development of the fungus occurred exclusively as mycelium formation, the level of polyunsaturated fatty acids (γ-linolenic and arachidonic acid) increased in the presence of trehalose and that of linoleic acid increased in the presence of nTAGs. These results may suggest that unsaturated fatty acids and membrane lipids are related to the cell wall formation and the implementation of morphogenetic programs in mucorous fungi.

Keywords: Mucor hiemalis, lipids, mycelium, trehalose, triacylglycerols, itraconazole

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A close relationship exists between lipid synthesis and the key processes involved in metabolism, growth, and survival in fungi. The role of lipids in the fungal life-sustaining activity (including morphogenesis) is considered in terms of their involvement in the cellular processes where they function as structural and storage compounds, adaptation factors under unfavorable environmental conditions, and regulatory agents [1–8]. The data available in the literature indicate that some lipids and fatty acid derivatives are universal regulators. They exert a multiple-factor influence on the morphogenesis of fungi that are pathogenic for plants and mammals [8–12]. Fungal morphogenesis, including the mycelium ↔ yeastlike cells transition, is accompanied by changes in the composition of phospholipids, free and esterified sterols, triacylglycerols, and fatty acids [13–17]. The use of azoles and polyene antibiotics made it possible to elucidate the role of the changes in the composition of sterols and other lipids for morphogenesis [18–32]. Moreover, there are data suggesting that the disaccharide trehalose should be considered a protective compound against various stress factors such as freezing, heating, and osmotic or oxidative stress [33–37]. It also produces a morphogenetic effect that, in contrast to that of glucose, results in exclusive development of the mycelial form of the fungus, without producing budding cells, even in the absence of oxygen in the medium [38]. The relationship between lipid composition and the capacity of the fungi of the genus *Mucor* for dimorphic growth is of considerable interest because these fungi are producers of a large number of biologically active compounds, while the mycelium and the yeast-like forms differ in terms of their productivity.

According to the data presented in the literature, the species *Mucor hiemalis* is considered monomorphic, i.e., capable of growth only in the form of mycelium [13]. Our studies revealed the heterogeneity of this species in respect to its capacity for dimorphism. Under the influence of environmental stress factors (the presence of chloroanilines, nutrient depletion, etc.) some representatives of the species *M. hiemalis* (the strain *M. hiemalis* F-1156) were also capable of yeastlike growth involving arthrospore formation [39–42]. In this work, the previously uninvestigated strain *M. hiemalis* VKM F-1431 was used as the model. It differs from *M. hiemalis* F-1156 in its pronounced capacity for yeastlike growth immediately upon sporangiospore germination.

The goal of this work was to investigate the growth and the lipid composition of the fungus *M. hiemalis* VKM F-1431 grown in the presence of morphogenetic effectors including those belonging to lipids or affecting their synthesis. We used the following substances: exogenous triacylglycerols (TAGs), the antibiotic itra-

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Table 1. The fatty acid composition of the sterilized (sTAGs; 0.5 atm, 110°C, 30 min) and unsterilized (nTAGs) triacylglycerol preparations used as morphogenetic effectors. The data are the percentages of the total fatty acids

Lipids	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$n9c	$C_{18:2}$n6c
nTAGs	7.04	4.3	19.6	69.04
sTAGs	14.10	7.23	49.48	29.19

conazole (an inhibitor of ergosterol synthesis), and trehalose.

MATERIALS AND METHODS

The sporangiospores of a 6-day *M. hiemalis* Wermer VKM F-1431 culture grown on wheat bran were washed off the surface of the sporogenic mycelium with sterile distilled water and used as inoculum on the basic liquid medium whose composition was as follows (g/L): glucose (carbon source), 60.0; urea, 2.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$ – 0.5; K_2HPO_4 – 1.0; $ZnSO_4 \cdot 7H_2O$ – 0.05; $FeSO_4 \cdot 7H_2O$, 0.01; yeast extract, 0.5; pH 7.0. The solutions of glucose, trehalose, and urea and the triacylglycerol preparation were sterilized separately (0.5 atm, 110°C, 30 min) and mixed with the mineral salt solution immediately before sporangiospore inoculation.

In some series of experiments, the disaccharide trehalose (60 g/L) that is responsible for protecting the membrane phospholipids under stress was used as the carbon source instead of glucose. In addition, we supplemented the basic medium with a number of compounds that are capable of morphogenetic effects such as (i) the antibiotic itraconazole, an inhibitor of the P450-dependent C14-demethylase involved in ergosterol synthesis (as DMSO solution yielding a final itraconazole concentration of 2 μg/mL in the medium) and (ii) membranotropic lipid compounds (sunflower oil TAGs with unsaturated $C_{18:1}$ and $C_{18:2}$; acyl chains; 30 mg per 50 mL of the medium). Fungal mycelium growth requires ergosterol and TAGs that are membrane and storage lipids, respectively. In this work, both sterilized (sTAGs) and unsterilized (nTAGs) triacylglycerol preparations were used. To prevent bacterial contamination (which was particularly important for experimental systems with itraconazole and nTAGs), a mixture of the antibiotics penicillin and streptomycin (10000 U and 10 mg/mL, respectively; Sigma, United States) was added.

Spore suspension with a density of 10^6 cells/mL was used as inoculum. Cultivation was carried out in 250-mL flasks with 50 mL of the medium on a shaker (130 rpm) at 27°C for 24 h (to reach the late trophophase).

Spore germination was monitored and cell morphology was examined with an Axio Imager.DI light microscope (Carl Zeiss, Germany) at a magnification of 400× in a phase-contrast system.

Lipids were extracted from the biomass by the modified Folch method [43], subjected to acidic methanolysis, and analyzed by GLC using a Chromatek Kristall-5000.1 chromatograph (Russia) on an Optima-240 60 m × 0.25 mm × 0.25 μm capillary column (Macheray-Nagel GmbH & Co, Germany). The stationary phase was 33% cyanopropyl–methyl–67% dimethylpolysiloxane; the system operated in a programmed mode. The column temperature was 130 to 280°C, the carrier gas (helium) consumption rate was 30 mL/min.

The composition of each of the lipid classes contained in the samples was determined by TLC on Kieselgel 60 F_{254} plates (Merck, Germany). The following solvent systems were used for chromatography: hexane–diethyl ether–acetic acid (80 : 20 : 2) for neutral lipids; chloroform–methanol–water (60 : 25 : 4) and chloroform–acetone–methanol–acetic acid–water (50 : 20 : 10 : 10 : 5) for polar lipids in the first and the second direction, respectively. Lipids on the chromatograms were developed with 10% phosphomolybdic acid solution in methanol with subsequent heating. Lipid identification on the chromatograms was performed by comparing their R_f with those of the standards (Sigma, United States) and by carrying out the qualitative tests. Densitometric analysis was performed using the Dens and Sorbfil software (Russia). Standard solutions of PC, TAG, and total fatty acids were used as the standards for plotting the calibration curves.

The results were processed with the Microsoft® Office Excel® 2007 software package. The mean square deviation was used to estimate the data dispersion.

RESULTS AND DISCUSSION

At the initial stage of our research, while preparing the cultivation medium, we noted a decrease in the fluidity of the TAG preparation (used as a morphogenetic effector) upon sterilization. This gave a basis to the suggestion that the fatty acid composition of the TAG could undergo substantial changes while heated during sterilization. For this reason, we conducted a comparative study of the fatty acid composition of sterilized and unsterilized TAG preparations. The results confirmed our suggestion. Changes in the acyl chains of the TAGs upon heating manifested themselves in a twofold decrease in the percentage of linoleic acid residues and an increase in the levels of the monoenoic oleic acid and saturated fatty acids (Table 1). We assumed that the changes in the fatty acid composition of the TAGs during sterilization could considerably affect the development of the fungus. It is known that a number of lipids, including fatty acid derivatives, are morphogenetic effectors. There-

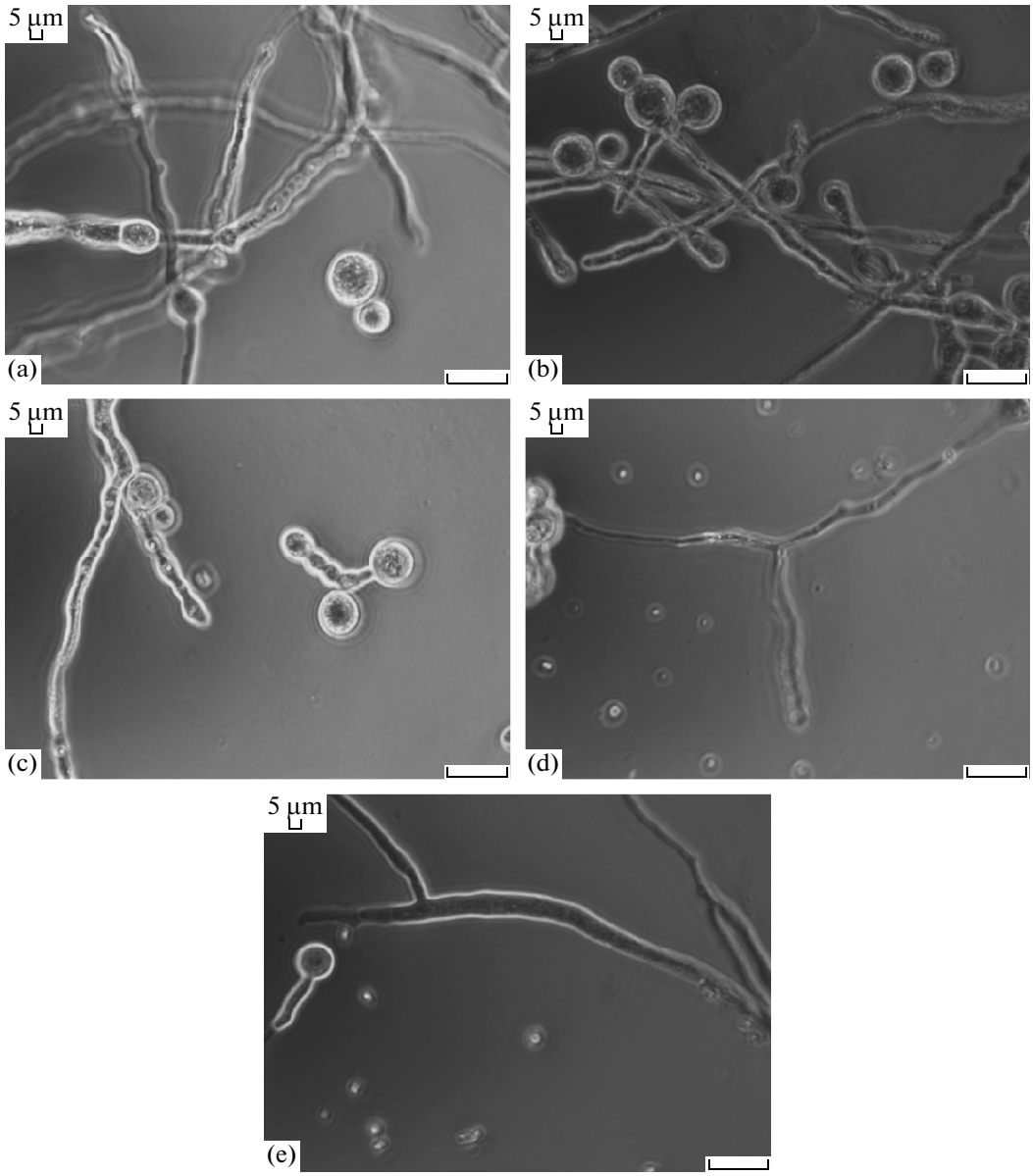

Fig. 1. Growth of *M. hiemalis* VKM F-1431 with: glucose (6%, control) (a); itraconazole (2 μg/mL) (b); sTAGs (0.6 mg/mL) (c); nTAGs (0.6 mg/mL) (d); and trehalose (6%) (e). Phase contrast system. Bar, 25 μm.

fore, we used preparations of both sterilized (sTAGs) and unsterilized, i.e., native (nTAGs) TAGs as lipid additives in our further studies.

Using sporangiospores from a 6-day *M. hiemalis* VKM F-1431 culture (arbitrarily called "young spores") as inoculum caused the cultures to develop in compliance with the mycelium strategy; nonetheless, we observed solitary yeastlike cells in the control system (with glucose, Fig. 1a) and in the systems with itraconazole (Fig. 1b) and sTAGs (Fig. 1c). The systems with trehalose and nTAGs lacked any yeastlike growth on day 1; the fungus grew exclusively in the form of mycelium (Figs. 1d and 1e, respectively).

The accumulation of mycelial biomass and its lipid content are shown in Table 2. Itraconazole caused a slight delay in sporangiospore germination followed by a rapid accumulation of biomass which marginally exceeded that in the control system. The maximum lipid accumulation was found in the nTAGs system.

The neutral lipids of the tested strain included mono-, di-, and triacylglycerols (MAGs, DAGs, and TAGs), free sterols (FSs), free fatty acids (FFAs), esterified sterols (ESs), and a number of unidentified minor fractions (X-1–X-4). The polar lipids contained phosphatidylethanolamines (PEAs), phosphatidylcholines (PCs), phosphatidic acids (PAs), diphosphatidylglycerols (DPGs, cardiolipins), gly-

Table 2. Biomass and lipid accumulation in *M. hiemalis* F-1431 mycelium grown with morphogenetic agents. Inoculum, 6-day sporangiospores. Late trophophase. The data are based on the results of three independent experiments

Experimental system	Control system	Trehalose	Itraconazole	nTAGs	sTAGs
Biomass, g/L	8.14 ± 0.52	7.51 ± 0.90	9.79 ± 1.46	8.88 ± 4.03	8.55 ± 0.88
Lipids, %	9.27 ± 2.06	8.55 ± 1.48	11.87 ± 6.51	21.45 ± 9.06	13.60 ± 2.33

Table 3. Total lipid composition of *M. hiemalis* F-1431 mycelium grown with morphogenetic agents. Inoculum, 6-day sporangiospores. Late trophophase. The data presented are based on the results of three experiments

Lipids	Control system	Trehalose	Itraconazole	nTAGs	sTAGs
PLs	10.30 ± 0.85	11.93 ± 1.59	9.65 ± 0.59	11.90 ± 1.82	12.73 ± 3.31
DAGs	10.10 ± 1.08	11.80 ± 2.17	12.5 ± 1.81	11.10 ± 0.49	10.67 ± 0.46
FSs	11.17 ± 0.69	12.73 ± 1.08	6.10 ± 0.62	7.93 ± 1.48	7.83 ± 0.62
X-1	5.67 ± 0.86	3.47 ± 1.08	5.55 ± 0.36	6.13 ± 2.22	3.87 ± 0.40
FFAs	11.57 ± 0.53	10.83 ± 2.87	12.93 ± 3.61	13.47 ± 3.84	11.73 ± 2.95
X-2	4.03 ± 0.28	4.90 ± 1.90	2.53 ± 0.76	2.43 ± 0.36	3.87 ± 0.83
TAG	30.27 ± 1.03	25.43 ± 1.64	32.60 ± 4.65	31.47 ± 8.66	32.87 ± 5.27
X-3	2.37 ± 0.17	Trace amounts	5.93 ± 2.19	5.70 ± 1.37	1.55 ± 0.08
ESs	14.53 ± 1.35	18.90 ± 10.12	12.00 ± 3.06	11.83 ± 3.60	15.10 ± 2.42

colipids (GLs), and a number of minor fractions (X-1–X-4). Among the fatty acids, those with 16 and 18 carbon atoms in the chains, with various numbers of double bonds, predominated.

Our research on the lipid composition of 1-day mycelium at the trophophase stage (Table 3) revealed that in the presence of itraconazole, an inhibitor of ergosterol synthesis, the free sterol level in the total lipid fraction was almost two times lower than in the control system (6.1 and 11.17%, respectively). The combination of seemingly contradictory data—a lowered free sterol level and a considerable mycelial growth at the experimental low itraconazole concentration (2 μg/mL)—testifies to an adaptive response of the fungus to this stress factor by enhanced biofilm formation. This subject will be addressed in a special study.

With trehalose in the cultivation medium, the lipid content and the TAG percentage were below those in the other tested systems. The polar lipids (PLs, Table 4) were characterized by a high phosphatidic acid (PA) content (47.6%) and a low PEA level (21.94%). The lowest ratio between the main membrane lipids (PEA/PC) was found in the systems with trehalose (1.48) and nTAGs (1.76), in contrast to the systems with itraconazole and sTAGs (2.63 and 3.20, respectively). This attests to a relatively high PC percentage and, accordingly, to a stable structure and a high functional state of the cell membranes. Comparison between the nTAGs and the sTAGs system reveals that the enhanced PEA/PC ratio in the mycelium correlates with the formation of yeast-like cells in the culture in the presence of sTAGs, which differ from nTAGs in terms of their fatty acid ratio.

Table 4. Polar lipid composition of *M. hiemalis* F-1431 mycelium grown with morphogenetic agents. Inoculum, 6-day sporangiospores. Late trophophase. The data are based on the results of three independent experiments

PLs, % of the total	Control system	Trehalose	Itraconazole	nTAGs	sTAGs
Gl-1	6.53 ± 1.02	0.87 ± 0.4	3.34 ± 1.59	2.85 ± 1.07	3.78 ± 0.87
Gl-2	1.27 ± 0.94	Trace amounts	2.55 ± 1.82	Not detected	Trace amounts
PAs	25.48 ± 9.96	47.60 ± 6.40	32.88 ± 4.20	23.58 ± 4.21	36.44 ± 12.07
Gl-3	3.85 ± 1.79	6.79 ± 3.15	4.33 ± 1.46	4.32 ± 0.08	1.91 ± 0.51
DPGs	3.86 ± 1.40	2.08 ± 1.70	5.67 ± 0.26	5.70 ± 0.92	2.25 ± 1.57
PEAs	36.44 ± 5.53	21.94 ± 6.88	29.45 ± 4.54	30.81 ± 4.56	39.83 ± 4.83
X-1	0.34 ± 0.01	Trace amounts	2.40 ± 1.70	1.59 ± 0.15	Not detected
PCs	19.06 ± 5.25	14.85 ± 4.07	11.19 ± 5.83	17.54 ± 0.05	12.45 ± 2.99
X-2	Trace amounts	Not detected	Trace amounts	2.37 ± 0.07	Not detected
PSs	Trace amounts	1.96 ± 1.02	2.56 ± 0.84	4.44 ± 0.42	2.71 ± 0.03
PIs	Trace amounts	6.51 ± 2.38	6.09 ± 2.14	4.71 ± 0.96	2.02 ± 0.28
PEA/PC	1.91	1.48	2.63	1.76	3.20

Apart from directly affecting their target enzymes, ergosterol biosynthesis inhibitors (azoles) influence the activity of a large number of other membrane-bound enzymes, membrane transport, respiration, fatty acid synthesis, and cell morphology. The data available in the literature indicate that prerequisites for fungal mycelial growth are ergosterol (E) and triacylglycerols (TAGs). Apart from functioning as membrane (ergosterol) and storage (TAG) components, they also act as signal compounds that induce polarized growth in dimorphic ascomycete and basidiomycete fungi [7, 19–25]. Our results indicate that this statement is valid for a representative of mucorous fungi.

Research on the fatty acid composition of mycelium lipids (late trophophase, Fig. 2) demonstrated that, with itraconazole, an inhibitor of ergosterol biosynthesis, the oleic acid level was decreased (16.3% vs. 22.6% in the control system) and the γ-linolenic acid level was increased (17.1% vs. 12.1% in the control system). The increase in the share of γ-$C_{18:3}$ acid points to its involvement in the adaptation to itraconazole. The addition of sTAGs caused virtually no changes in the fatty acid composition of the lipids, compared with the control. With nTAGs, the palmitic and γ-linolenic acid levels significantly decreased (down to 16% and 10.5%, respectively) with a concomitant increase in the percentages of linoleic acid (37% vs. 18.5% in the control system) and $C_{20:2}$. The fatty acid composition of the lipids of trehalose-grown culture differed from all other systems, including the control: the level of the monoenoic oleic acid was twofold decreased (11%) and that of the trienoic γ-lino-

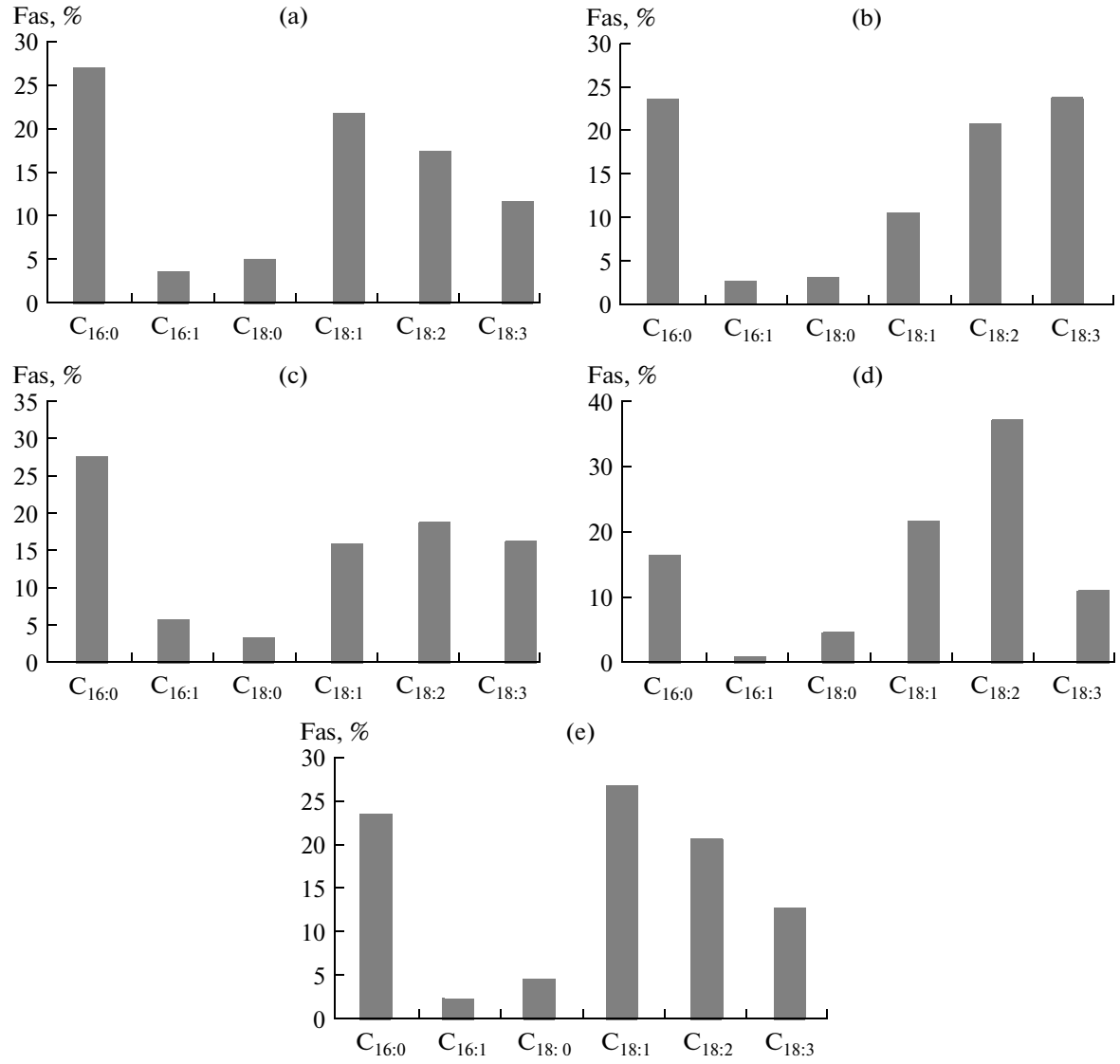

Fig. 2. The fatty acid composition of *M. hiemalis* VKM F-1431 mycelium grown with morphogenetic agents: glucose (6%, control) (a); trehalose (6%) (b); itraconasole (2 μg/mL) (c); nTAGs (0.6 mg/mL) (d); and sTAGs (0.6 mg/mL) (e). Inoculum, 6-day sporangiospores. Late trophophase. The data are based on the results of three independent experiments.

lenic acid was twofold increased (24.7% vs. 12.1% in the control system with glucose). Besides, the lipids contained low amounts of arachidonic acid ($C_{20:4}$; 0.7%), which was lacking in the control system. The desaturation degree (number of double bonds per 100 fatty acid molecules) of the lipids correlated with the morphogenetic features of the tested systems. The lowest values were characteristic of the systems in which yeastlike cells formed. The desaturation degree was 99.55 in the control system, 112.62 with itraconazole and 110.54 with TAG. The highest degree was detected in the systems with trehalose (131.33) and nTAGs (127.61).

Hence the low ratio between the main membrane lipids (PEA/PC) in the presence of trehalose and nTAGs in the medium attests to a relatively high PC percentage and, accordingly, to a stable structure and a high functional state of the membranes. Moreover, elevated levels of the polyunsaturated γ-linolenic and arachidonic fatty acids with trehalose or of linoleic acid with nTAG correspond to exclusively mycelial development of the fungus. This suggests a relationship between the dynamics of unsaturated fatty acids and membrane lipids and the implementation of morphogenetic programs by the fungi.

According to the data presented in the literature, a prerequisite for the manifestation of dimorphism is the presence of a fermentable hexose (glucose) in the medium [44, 45]. It is also known that the disaccharide trehalose sustains only the mycelial growth of the fungi without causing budding cell formation, even in the absence of oxygen in the medium [38]. Trehalose is considered a protective compound [33–35]. The data obtained concerning the lipid composition confirm the idea that trehalose, being a membrane protector, sustains hyphal growth. This idea has repeatedly been put forward in the literature.

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