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EXPERIMENTAL ARTICLES

Changes in Growth Patterns and Intracellular Calcium Concentrations in *Aspergillus awamori* Treated with Amphotericin B

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Abstract—Growth patterns and intracellular Ca^{2+} concentrations in the mutant strain *Aspergillus awamori* 66A containing a recombinant aequorin gene were studied in the presence of a permeabilizing fungicidal agent amphotericin B. The cell response, i.e., changes in the growth and development of the fungus (initiation of spore germination, mycelial growth, and intensity of sporulation) was dose-dependent. Low concentrations of amphotericin B (2.5 μM) stimulated spore germination: the number of germinating spores was 2–3 times higher than in the control (without the fungicide). At higher amphotericin concentrations (20 μM) spore germination was inhibited. Amphotericin B had a dose-dependent effect on mycelial growth and sporulation intensity on solid Vogel medium. Intracellular Ca^{2+} concentrations in the presence of amphotericin B were investigated using the luminescence of the photoprotein aequorin. High concentrations of amphotericin B (10 and 20 μM) were shown to cause an instantaneous increase in Ca^{2+} concentrations compared to the control and lower amphotericin concentration (2.5 μM). Ca^{2+} concentrations remained elevated throughout the experiment and correlated with the inhibition of mycelial growth and development.

Keywords: *Aspergillus awamori,* recombinant aequorin, permeabilizing fungicide, amphotericin B, cell response, sporulation, Ca^{2+}, signaling, Ca^{2+} dynamics

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An important line of activity in plant protection against phytopathogenic microflora, including the phytopathogenic micromycetes, is the search for fungicidal compounds. Treatment of barley seeds with preparations of microbial origin produces good results providing for decreased numbers of mold fungi [1]. At the same time, occurrence of micromycetes of the genus *Aspergillus*, which are potentially pathogenic for humans, remains high [2]. Toxicity of the members of this genus and their pronounced resistance to various fungicides motivated the search for new biologically active compounds; among them, amphotericin B, active against a number of phytopathogenic fungi (which cause many diseases, including those incurable by other antifungal preparations), turned out to be a promising candidate [3–5].

Amphotericin B belongs to the group of polyene antibiotics. It can increase membrane permeability for ions [6, 7], including calcium, which performs signaling functions for the control of a number of processes in a eukaryotic cell. The signaling functions of calcium in animals and plants are well-studied [8, 9]. In mycelial fungi, signaling functions of Ca^{2+} in development of apoptosis are known [10, 11]. However, in modern physiology, many issues in calcium signaling under the impact of stress—when impaired calcium homeostasis may affect the functioning of Ca^{2+} signaling systems, physiological processes, and maintenance of cell viability—remain unclear. Several scenarios of events are possible in case of impaired calcium homeostasis in a cell: they may cause toxicity through deregulation of calcium on one hand and, on the other, turn on the damage reparation processes, where Ca^{2+} plays a defining role as a mediator in signal transduction cascades [12]. Realization of one or another program depends on the extent to which Ca^{2+} homeostasis is imbalanced in a cell subject to certain physiological stimuli. To determine the possible scenarios of stress response, including the efficiency of fungicide therapy, a test system that would allow for analysis of the micromycete growth and development depending on the level of intracellular calcium is required.

The goal of the present work was to investigate the relationship between the growth and development of an *Aspergillus awamori* mutant strain containing a recombinant aequorin protein and changes in Ca^{2+} content in the fungal cytosol caused by the micromycete treatment with a permeabilizing fungicide amphotericin B.

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MATERIALS AND METHODS

The mutant strain *Aspergillus awamori* 66A expressing a light-dependent protein aequorin (kindly provided by the Laboratory of Micromycete Cell Investigation, Heriot-Watt University, Edinburgh) was the subject of the study [13].

The strain *A. awamori* 66A was cultured at 30°C in a liquid Vogel medium supplemented with sucrose (VS medium) and on a solid (2% agar) medium of the same composition enriched with glucose (GEM) [14]. Experiments were performed on a monospore culture of *A. awamori* 66A which was obtained from a conidial suspension of the fungus with spore concentration of 20–30 spores/mL. On the surface of the liquid Vogel medium, the fungus grew as individual colonies, which were then transferred onto the solid medium after 24-h incubation. After 7 days of growth of the surface culture, the spores were washed off with 0.8% NaCl solution (PZ), cleared from the residual medium and mycelium (centrifugation at 3000 *g* for 15 min), resuspended in PZ solution, and stored at 4°C. Spore concentration was determined by counting in a Goryaev chamber. The density of spore suspensions used in the experiments was 3×10^5 spore /mL.

The fungicide amphotericin B (Sigma), a polyene antibiotic synthesized by actinomycetes of the genus *Streptomyces*, also known under the names of Amfostat, Amphotericin B, Fungilin, Fundizone, etc. [3], was used as a stress factor. The effect of amphotericin on the development of *A. awamori* was determined upon its introduction into the micromycete culture at various growth phases.

The effect of amphotericin on the process of spore germination was assayed by the rate of their swelling and the number of spores with germ tube. Amphotericin concentrations were chosen considering the fact that high doses of the antibiotic have a fungicidal effect on the micromycete, while intermediate doses produce a static effect, with the reproductive functions blocked, while cell viability is preserved. To study the fungicidal effect, amphotericin was used at concentrations of 2.5, 10, and 20 μM. Spore swelling was analyzed in a drop of liquid Vogel medium (10 μL) on a slide at 3×10^5 spore/mL and amphotericin concentrations from 2.5 to 20 μM per sample. To avoid drying, the slides with spores were placed in a humid chamber (100% humidity) and stored there at 30°C for 6 h. Spore diameter (d, μm) in the experimental and control (grown on fungicide-free medium) samples was determined every 2 h using an ocular micrometer (Axio Imager 2 microscope, Zeiss).

Spore germination was studied upon incubation at 30°C in a liquid Vogel medium in tubes at spore concentration of 3×10^5 spore/mL and amphotericin concentrations of 2.5, 10, or 20 μM. During the 24-h incubation, the number of spores with a germ tube (beginning of the lag phase) was registered and branching of the germ tube was determined (beginning of the exponential phase). The number of spores with a germ tube and branching of the hyphae from the main germ tube were counted in a Goryaev chamber in 20 fields of view.

The effect of the fungicide on the quality of submerged *A. awamori* culture used as inoculum was determined from the rate of mycelium growth on a solid Vogel medium (colony radius, mm). For this purpose, spores (3×10^5 spore/mL) were inoculated into liquid Vogel medium and incubated for 24 h (until the early exponential phase). Amphotericin was added to the grown *A. awamori* culture at various concentrations (2.5, 10 and 20 μM) and incubation continued for another 24 h; then aliquots of the culture (0.1 μL) were plated onto solid medium and incubated at 30°C for 7 days. The radius of micromycete colonies was measured every 24 h and the total and specific growth rates were calculated according to the technique described in [15].

The effect of amphotericin on spore formation by *A. awamori* was studied under the experimental conditions described above. One-day culture of *A. awamori* grown in tubes with Vogel medium containing amphotericin (2.5, 10, and 20 μM) was plated (0.1 μL) onto the solid Vogel medium. During 7 days of growth, blocks of agar culture along the colony circumference were cut out with a laboratory drill every 24 h, placed into a liquid medium, and shaken (15 min, 200 rpm) to free the spores. The number of spores per 1 mL of suspension was counted in a Goryaev chamber.

The effect of amphotericin on calcium content in the cytosol of *A. awamori* cells was determined by the luminescence of recombinant aequorin [16]. Aequorin luminescence was measured in the cells of the culture grown in liquid Vogel medium in a 96-well plate as described previously [17]. One hundred microliter spore suspension at starting concentration of 5×10^5 cells/mL was introduced into each well and incubated at 30°C for 24 h till the onset of the exponential growth phase. Then, coelenterazine was introduced into the cultures and they were incubated for 4 h to reduce aequorin. Then, amphotericin was added to each cell at concentration of 2.5, 10, or 20 μM (equal volumes of water were added to the control samples) and luminescence was measured with a luminometer for 10 min and expressed in relative light units (RLU). Since light emission was not high, a special luminometer equipped with a photomultiplier (EG & G Berthold LB 96 P Multilumat) was used to measure it.

The results were processed by variation statistics analysis with reliability criterion $P < 0.05$.

RESULTS

In the study of the effect of amphotericin on spore germination and *A. awamori* culture growth, optimal spore concentration in the experiments was 3×10^5 spore/mL

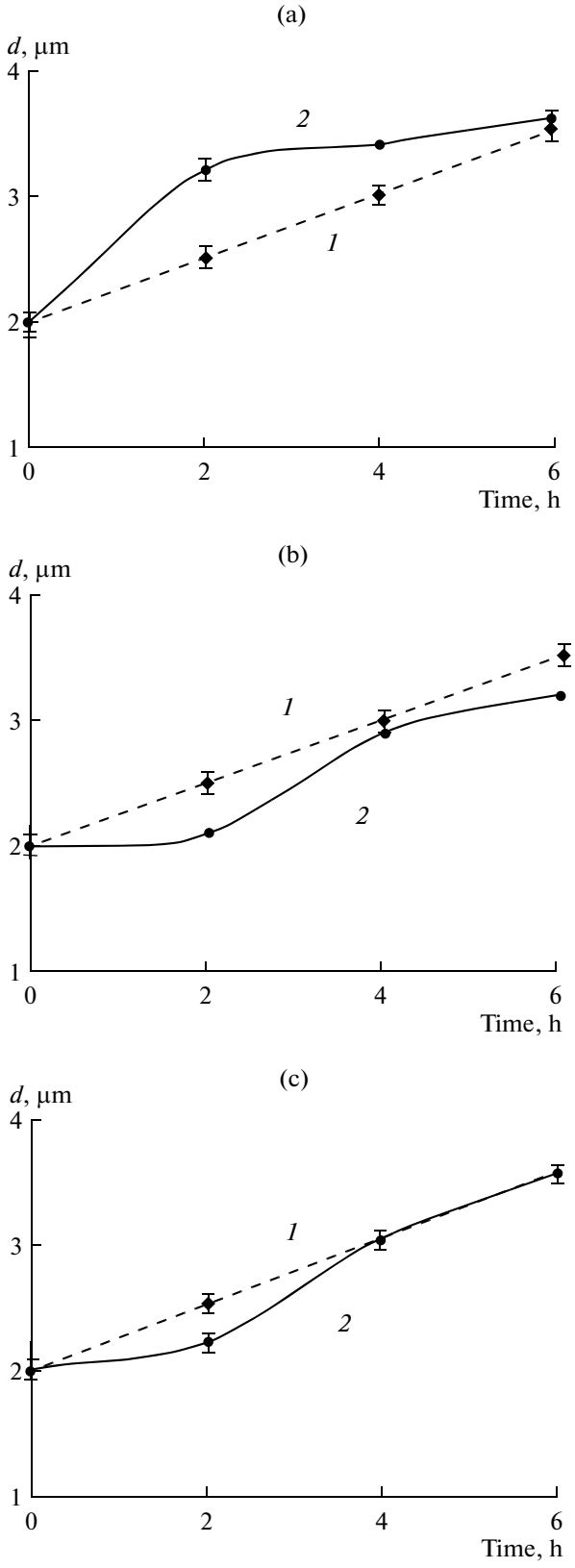

Fig. 1. Dynamics of *A. awamori* spore swelling (*d*, μm) upon incubation in Vogel medium containing amphotericin: (*1*), fungicide-free medium, (*2*), experimental samples with the fungicide at doses (a) 2.5, (b) 10, and (c) 20 μM, respectively.

liquid Vogel medium. Spore suspensions of higher density could not be used due to spore agglomeration and formation of hyphae intertwining upon their germination.

The effect of amphotericin on *A. awamori* spore germination was judged by the change in their volume upon swelling due to metabolism of the endogenous and exogenous substrates at the initial stage of fungal development. Amphotericin at concentrations of 10 and 20 μM suppressed spore swelling during 2 h incubation, although this effect was neutralized by the end of observation (6 h) (Figs. 1b, 1c). On the contrary, at low concentrations (2.5 μM), amphotericin activated the processes of spore swelling: after 2 h incubation, spore diameter increased by 28% if compared to the control; although this effect disappeared by the end of the experiment (Fig. 1a). It should be pointed out that growth-stimulatory effects of antibiotics at low concentrations (1/5–1/10 to their bactericidal dose) have been reported. For example, streptomycin stimulated growth of *Streptomyces griseus* at concentration of 100 μg/mL and suppressed it at 500 to 1000 μg/mL [18]. Microscopic analysis of *A. awamori* spore germination after 12 h of incubation in liquid Vogel medium evidenced the appearance of germ tubes in some spores of the control sample. In the experimental variants, the fungicide at a concentration of 20 μM suppressed formation of germ tubes insignificantly, while upon decrease in its concentration (<10 μM) it stimulated spore germination in a dose-dependent manner (Fig. 2). It should be noted that the inhibitory effect of amphotericin was less pronounced at the stage of *A. awamori* spore germination than at the stage of spore swelling.

In the next series of experiments, the effect of amphotericin on the micromycete development was studied by introduction of the fungicide in the cultures at the beginning of the exponential growth phase (24 h) at concentrations of 20, 10, and 2.5 μM and subsequent incubation. After 6, 12, 18, and 24 h, the number of lateral hyphae stemmed from the main germ tube was counted. In both control and experimental samples, the number of branching spores increased with increased incubation time. Amphotericin stimulated the formation of lateral hyphae at low concentrations (2.5 μM), although inhibited branching at concentrations of 10 and 20 μM (Table 1).

Since inoculum quality affects the development of a microbial culture, in the next series of the experiments we used *A. awamori* cultures grown in the presence of various doses of the fungicide as the starting ones. In the experiments, the antibiotic (2.5, 10, or 20 μM) was added to the culture grown for 24 h in liquid medium and the aliquots were inoculated onto agarized Vogel medium and incubated for 7 days. The effect of the fungicide on the quality of the inoculum was judged by the change in the size of colonies of the growing micromycete (*d*, μm), which was used to calculate the growth rate of *A. awamori* mycelium. It turned out that the size of the colonies in the samples

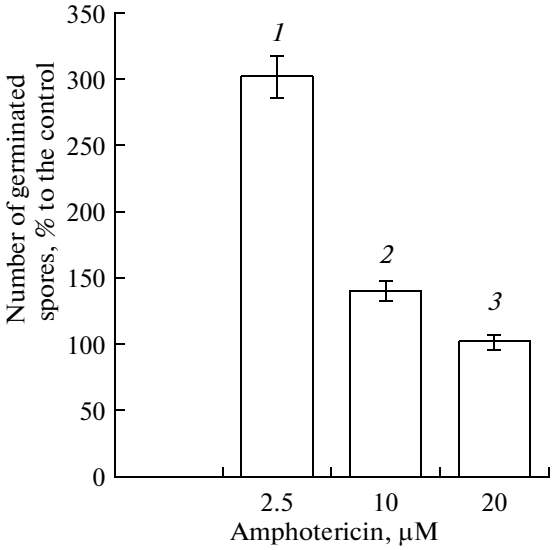

Fig. 2. Dynamics of spore germination in liquid Vogel medium containing amphotericin: 100%, the amount of germinated spores in the control (fungicide-free medium); (*1*), (*2*), and (*3*), number of spores with the germ tube after 24-h incubation in the medium containing amphotericin at concentrations of 2.5, 10, and 20 μM, respectively.

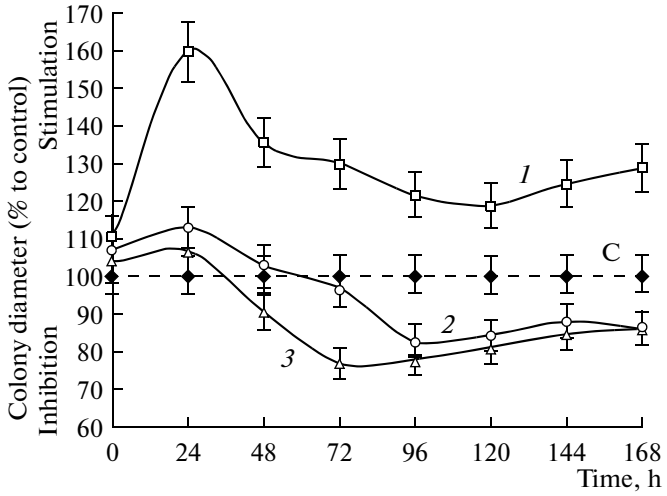

Fig. 3. Dynamics of *A. awamori* colony growth on solid medium upon the effect of various concentrations of amphotericin on the inoculate: C, control colony diameter (amphotericin-free medium) considered as 100%; (*1*), (*2*), and (*3*), concentrations of amphotericin of 2.5, 10, and 20 μM, respectively.

treated with 2.5 μM fungicide exceeded that in the control samples. Under the effect of 10 and 20 μM, the colonies were smaller than in the control samples (Fig. 3). Effect of a stimulating dose of the fungicide (2.5 μM; exceeding the control level by 60–40%) was more pronounced during the first day of cultivation; although it decreased after 48 h, it still exceeded the control level throughout the whole period of observation. This correlates with an increase in the specific growth rate of *A. awamori* culture (Fig. 4b) to maximum values 24 h after plating of the aliquots of micromycete culture grown in the presence of amphotericin (2.5 μM) onto agarized medium, which is typical of an exponentially growing culture. In the variants with fungicide concentration of 10 μM (Fig. 4c), specific growth rate of the culture was as high as in the variant with 2.5 μM after 24 h. Upon further increase in the antibiotic concentration (20 μM) in the cultures used as inocula, suppression of colony growth and inhibition of specific growth rate were observed (Fig. 4d).

Study of the dose-dependent effect of amphotericin on spore formation by *A. awamori* (Table 2) showed that the number of spores in the control and experimental samples of surface cultures increased after 7 days. Under the effect of high doses of amphotericin (20 and 10 μM), spore formation was suppressed starting from the second day of cultivation. At the same

Table 1. Dynamics of hyphae branching in the microcolonies of *A. awamori* after treatment of a 24-h culture with amphotericin

Duration of fungicide treatment, h	Number of hyphae in a microcolony							
	control*		experiment, fungicide-containing medium					
			2.5 μM		10 μM		20 μM	
	number of hyphae	% to the control	number of hyphae	% to the control	number of hyphae	% to the control	number of hyphae	% to the control
0	1.13 ± 0.05	100	1.15 ± 0.06	101.76	1.16 ± 0.07	102.65	1.16 ± 0.07	102.65
6	3.50 ± 0.50	100	5.10 ± 0.50	145.71	3.60 ± 0.50	102.85	3.10 ± 0.50	88.57
12	4.60 ± 0.80	100	6.40 ± 0.70	139.13	4.70 ± 0.30	102.17	3.40 ± 0.70	73.91
18	5.90 ± 1.00	100	7.60 ± 1.20	128.81	4.90 ± 0.40	83.05	3.90 ± 0.80	66.10
24	6.60 ± 1.20	100	8.90 ± 1.40	134.84	4.70 ± 0.20	71.21	2.90 ± 0.40	43.93

* Cells of the culture not treated with amphotericin were used as the controls. The results are mean values ± standard deviation (number of repeats $n = 7$).

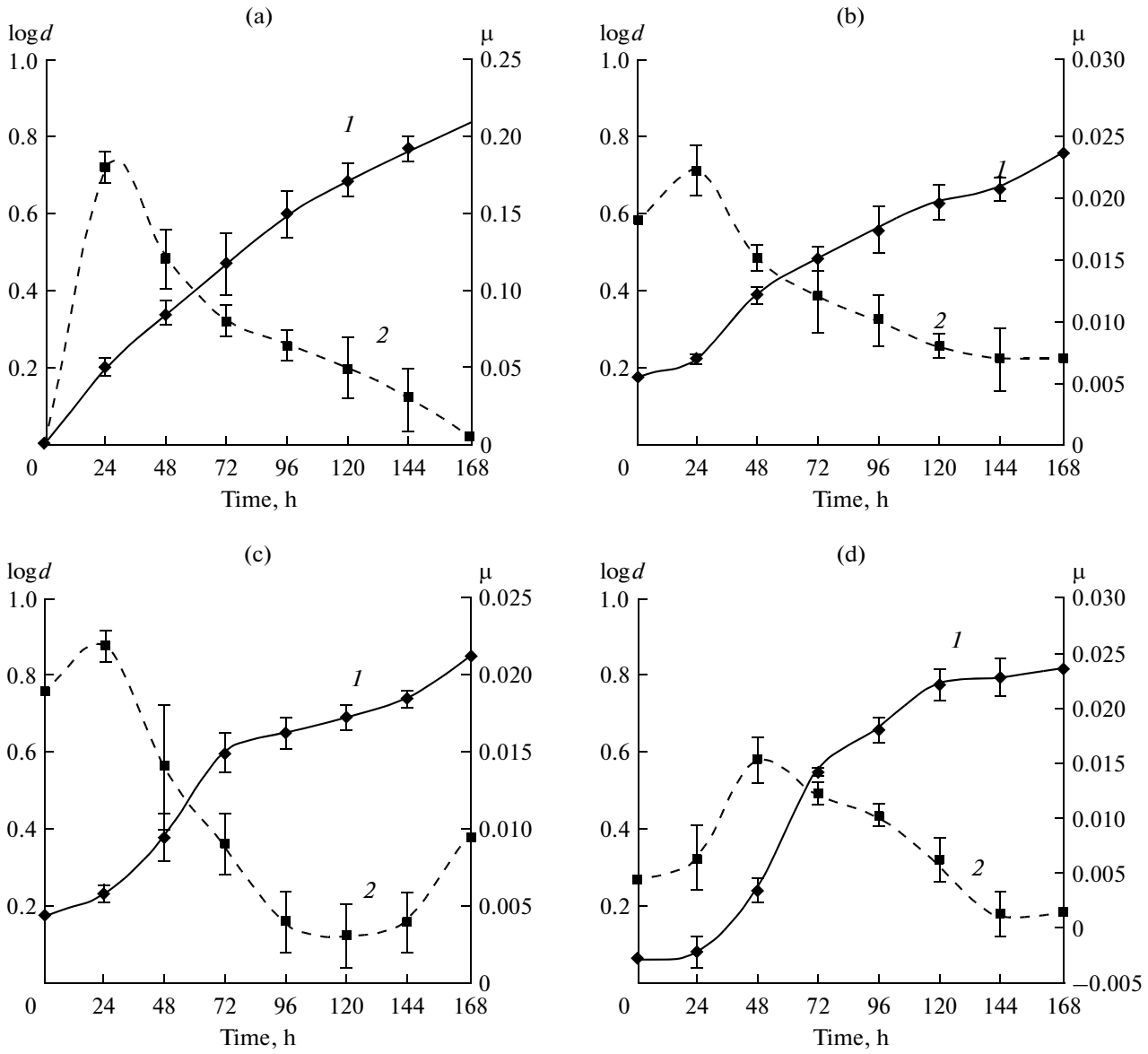

Fig. 4. Changes in specific growth rate (μ, h^{-1}) of the *A. awamori* culture depending on the amphotericin concentration (μM): control, fungicide-free medium (a); 2.5 (b); 10 (c); and 20 (d); (*1*), log *d*, colony diameter; and (*2*), specific growth rate of the colony (μ, h^{-1}).

time, the process was stimulated by a lower (2.5 μM) dose of the antibiotic.

Thus, investigation of the cell response to the effect of amphotericin indicated dose-dependency of the fungicide effects at all stages of *A. awamori* culture development (initiation of spore germination, mycelium growth, and intensity of sporulation).

At the next stage of the work, we studied the effect of amphotericin on the formation of Ca^{2+} response in the spores of the micromycete grown in liquid Vogel medium in plate wells. Measurement of the intracellular calcium concentration was possible due to the use of a mutant strain *A. awamori* 66A carrying a recombinant gene of *Aequorea victoria* responsible for synthesis of a Ca^{2+}-dependent light-sensitive protein aequorin (21400 Da). Aequorin is known to comprise a single polypeptide chain, apoaequorin, a hydrophobic luminophor, coelenterazine, and bound oxygen [16]. As a result of a Ca^{2+} ion binding to two out of three calcium-binding sites in aequorin, the protein exhibits oxygenase properties, catalyzing the oxidation of coelenterazine with the bound oxygen. This results in luminescence of aequorin (emission of blue light, λ_{max} 470 nm), whose intensity depends on concentration of free calcium ions.

In the experiments, the fungicide was introduced into 24-h cultures of *A. awamori* at concentration of 2.0 to 20 μM. It turned out that the concentration of

Table 2. Intensity of sporulation by surface cultures of *A. awamori* pre-incubated in the media containing various doses of amphotericin for 24 h

Age of the surface culture, days	Number of spores per 1 mL suspension, $\times 10^8$			
	Fungicide concentration, μM			
	control	2.50	10.00	20.00
0	3.00 ± 0.50	3.30 ± 0.06	3.20 ± 0.07	3.10 ± 0.07
1	3.20 ± 0.10	5.10 ± 0.50	3.60 ± 0.50	3.40 ± 0.50
2	4.00 ± 0.20	5.40 ± 0.70	4.10 ± 0.30	3.60 ± 0.70
3	5.10 ± 0.80	6.60 ± 1.00	4.90 ± 0.40	3.90 ± 0.80
4	5.70 ± 0.20	6.90 ± 0.40	4.70 ± 0.20	4.40 ± 0.40
5	6.10 ± 0.80	7.20 ± 0.20	5.10 ± 0.07	4.90 ± 0.40
6	6.30 ± 0.50	7.80 ± 0.30	5.50 ± 0.10	5.30 ± 0.20
7	6.56 ± 0.70	8.39 ± 0.10	5.60 ± 0.08	5.60 ± 0.10

Time of spore suspension inoculation onto dense medium is indicated as 0. Control was the culture not treated with the fungicide. The results are mean values ± standard deviation (number of repeats $n = 6$).

2 μM was the minimum for reliable induction of increased calcium content in the cytoplasm of *Aspergillus* cells (Fig. 5). Increase in the dose of the permeabilizing fungicide led to an increase in the intracellular calcium concentration.

In the study of the effect of amphotericin on kinetics of Ca^{2+} response generation, its concentration in the cells of the control and experimental samples was monitored for 6 min. Measurements of the dynamics of Ca^{2+} demonstrated a drastic increase in its content already 1 min after the introduction of amphotericin into the experimental cultures (Fig. 6). Both the intensity and rate of Ca^{2+} increase in the cytoplasm were dose-dependent. Upon the addition of low concentrations (2.5 μM) of amphotericin, Ca^{2+} response was formed more slowly and was less pronounced than upon the effect of high doses of the fungicide (10–20 μM). Dose-dependency of the effect of amphotericin was registered through the differences in (1) time required for an increase in Ca^{2+} concentration in the cytosol; (2) amplitude of the rise; and (3) duration of the elevated level of calcium in the cytoplasm.

DISCUSSION

In fungi, Ca^{2+} transport is carried out by the system of pumps and channels located in the plasma membrane or in the membranes of the cell organelles storing Ca^{2+}, which may consume or release Ca^{2+} ions.

In the studies of Ca^{2+} signal regulation in the cells, much attention is paid to the role of mitochondria [19], in particular, of the mitochondrial carriers, such as calcium uniporter providing for Ca^{2+} consumption, and antiporters, necessary for the cation release from the organelle. For the cells of the yeast *Endomyces magnusii*, a mechanism of slow calcium release was demonstrated in the mitochondria [10]. Calcium release occurs in the absence of damage to the inner mitochondrial membrane (formation of a Ca^{2+} pore) and is performed by an electroneutral $Ca^{2+}/2H^+$ exchanger. According to the authors' data, the calcium transporting system, comprising independent ways of its accumulation and release, is involved in the regulation of the level of intramitochondrial calcium and modulation of oxidative metabolism in yeast cells via Ca^{2+}-dependent activation of NAD-dependent isocitrate dehydrogenase.

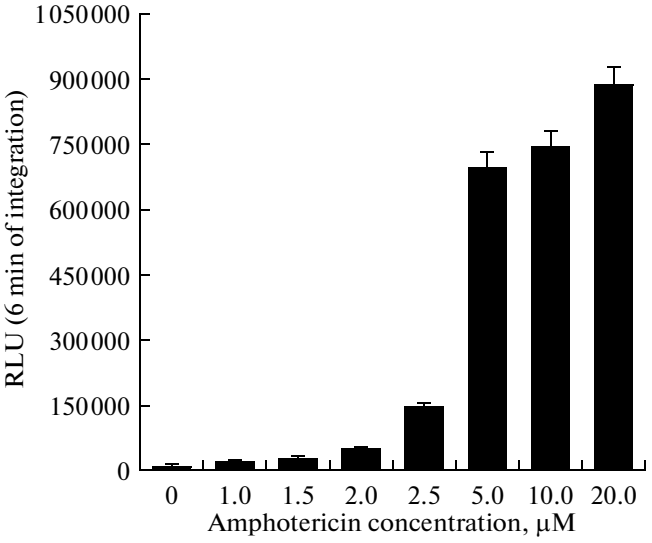

Fig. 5. Effect of amphotericin on the level of cytosolic Ca^{2+} expressed as levels of aequorin luminescence. The results present the mean ± standard error; RLU, relative light units.

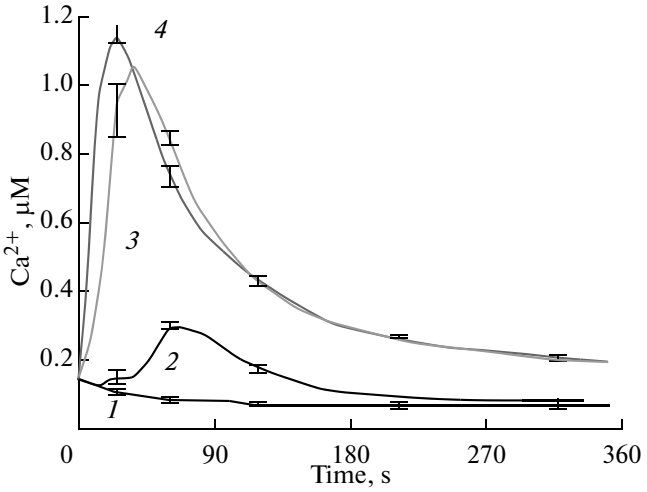

Fig. 6. Effect of amphotericin on the dynamics of Ca^{2+} response in *A. awamori* cells. Time of a single cycle is 11.6 s. Concentrations of amphotericin (μM): (*1*), 0 (control); (*2*), 2.5; (*3*), 10; and (*4*), 20. The results are mean ± standard error. The experiments were performed using the repeated measurements protocol.

Earlier, using various blockers of Ca^{2+} channels and agonists specifically increasing their activity, we found that Ca^{2+} bursts in the cells of *A. awamori* induced by physicochemical stimuli are regulated by an influx of Ca^{2+} from both the environment and intracellular reservoirs [17]. Also, factors of intercellular communication, particularly, alkylhydroxybenzenes (AHB), synthesized by a number of bacteria and yeasts, were shown to participate in the regulation of Ca^{2+} transport in the micromycete [20]. Alkyloxybenzenes, as well as amphotericin, exhibit membranotropic properties [21]. A hydrophobic long-chain homologue (C6-AHB) induced an increase in Ca^{2+} content in the cytosol of *A. awamori* with prolonged maintenance of the elevated Ca^{2+} level [22]. In other experiments, the AHB homologue was shown to function as a factor of intercellular communication acting as an alarmone, or an alarm signal imitating the stress impact [23]. Its effect on the physiological activity of microorganisms, as well as the effect of amphotericin, was dose-dependent; it stimulated the activity of test microorganisms at low concentrations and inhibited it at high concentrations [24]. The revealed relationship between an increase in the Ca^{2+} pool in *A. awamori* cells treated with amphotericin with the dose-dependent inhibition of growth and development of the micromycete is similar to the effects of long-chain alkylhydroxybenzenes. These data raise an issue of further studies on the effect of amphotericin as a microbial metabolite involved in microbial cell communication at the level of a microbial community.

The revealed changes in calcium pool in *A. awamori* cells under stress effects of amphotericin are caused by properties typical of polyene antibiotics, which interact with the membrane lipids with formation of pores for ions and non-electrolytes. Thus, interaction of an antibiotic filipin, which shares many features with amphotericin, with supported phospholipid bilayer membranes promoted a 10^4–10^5-fold increase in membrane permeability. At low concentrations of filipin (10^{-7} M), individual ion pores were observed in the membranes, while upon combined treatment with filipin and amphotericin B open ion pores were detected [25]. Therefore, our results demonstrating the effect of growth-inhibitory doses of a fungicide on the increase in the Ca^{2+} level in the cytosol of *A. awamori* agree with the literature data on the ability of amphotericin, as well as of other membranotropic compounds, to induce changes in the permeability of cell membranes for ions.

One of the paradoxes of calcium signaling systems is the fact that, on one hand, increase in the cellular calcium pool is required upon stress impact for signal generation and, on the other, prolonged maintenance of high calcium concentration in the cytoplasm may be lethal for a cell due to impairment of its energetics by binding of calcium with phosphate ions [26]. The demonstrated inhibition of growth and development of the fungus *A. awamori* by high doses of amphotericin correlates with the increase and prolonged maintenance of high Ca^{2+} levels in the cytosol of this micromycete. The revealed correlation of the growth-inhibitory effect of amphotericin and Ca^{2+} response of the micromycete cells makes the development of a test system to screen for compounds with fungicide activity based on the *A. awamori* mutant strain with recombinant aequorin promising.

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