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EXPERIMENTAL ARTICLES

Elemental Composition of Dormant and Germinating Fungal Spores

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Abstract—For dormant (spores 0) and germinating fungal spores (spores G), elemental composition and the K/Ca and P/S ratios were determined. According to the working hypothesis, the latter reflected the specifics of the spore physiological state. Mycelial fungi with different rates of spore transition from the exogenous dormant state in the absence of nutrients in reactivation media were studied. Carbon content in spores 0 correlated with the level of cellular lipids. The K/Ca ration in spores 0 was lower for *Aspergillus tamarii* and *Cunninghamella echinulata* than for *Aspergillus sydowii* and *Umbelopsis ramanniana*. The P/S ratio in *Aspergillus* dormant spores was lower than in zygomycete fungi, while in rapidly germinating spores of *A. tamarii* and *C. echinulata* this ratio was 1.5–1.75 times lower than in slowly germinating spores of *A. sydowii* and *U. ramanniana* strains. Thus, low K/Ca and P/S ratios in dormant fungal spores may be used to predict their more rapid transition from the dormant state, which is important in the case of mycelial fungi producing compounds used in biotechnology, as well as for the clinically significant strains.

Keywords: X-ray microanalysis, elemental composition, spores, dormancy, germination, *Aspergillus tamarii*, *Aspergillus sydowii*, *Umbelopsis ramanniana*, *Cunninghamella echinulata*

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In microorganisms, a dormant state is a strategy aimed at survival under unfavorable growth conditions (Lennon and Jones, 2011). Several types of dormancy are known for mycelial fungi, with constitutive and exogenous dormancy being the major ones. Constitutive (endogenous, deep) dormancy involves a complex multistage germination regulation (cytoplasmic regulation), when the presence of water alone is insufficient for cell transition to the metabolically active stage (Feofilova, 2003). The second type, exogenous dormancy, involves simpler regulation, and removal of limitation (usually the absence of exogenous water) is sufficient for the onset of germination. This dormancy type occurs in fungal vegetative spores, e.g., conidia and sporangiospores. Ability of fungal spores to germinate and the rate of this process depend both on the state of the intracellular components and metabolic systems and on environmental factors. Investigation of variations in the content of biogenic elements in the spores with exogenous dormancy transferred into starvation medium for germination is of special interest. Previous studies revealed ability to germinate under these conditions for the spores of some ascomycetes and zygomycetes (Mysyakina et al., 2016a).

X-ray microanalysis is one of the methods applicable for analysis of ionic homeostasis in various biological objects (Stewart et al., 1980; Pitryuk et al., 2002; Nagata, 2004). It was used, for example, to determine the differences in the content of individual biogenic elements and their pairwise ratios (S, P, Ca, and K; Ca/K and P/S) in the cells of various microorganisms (*Bacillus cereus, Micrococcus luteus*, *Saccharomyces cerevisiae*, *Mucor hiemalis*), which differed in their metabolic activity and proliferative ability from vegetative cells to viable dormant forms to nonviable cells (Mulyukin et al., 2002) or from old to young spores (Mysyakina et al., 2014). The differences in the content and ratio of biogenic elements in the dormant forms reflected the differences in their ionic homeostasis and metabolic level occurring on transition to the anabiotic state. They may be used to develop the diagnostic criteria of microbial physiological state. X-ray microanalysis provides information on the ratio of elements in individual cells, including spores, making it possible to obtain statistically reliable indices characterizing the state of the culture as a whole.

The goal of the present work was to investigate the elemental composition and ratios on transition of ascomycete and zygomycete fungal spores from the exogenous dormant state in the absence of salts and nutrients in the medium (distilled water).

MATERIALS AND METHODS

Fungal strains. The following strains with different growth rates on solid media (Mysyakina et al., 2016a) from the VKM collection were used as research subjects:

(1) VKM F-64—*Aspergillus tamarii* (Kita 1913) with a relatively high growth rate (the colony diameter up to 70 mm after 7 days of cultivation on CA medium at 25°C);

(2) VKM F-441—*Aspergillus sydowii* (Bainier et R. Sartory 1913) Thom et Church 1926, a slowly growing *Aspergillus* strain (the colony diameter not exceeding 30 mm after 7 days of cultivation on the wort agar medium at 25°C);

(3) VKM F-582—*Umbelopsis ramanniana* (Moeller 1903) W. Gams 2003 (synonyms: *Mucor ramannianus* Moeller 1903; *Mortierella ramanniana* (Moeller 1903) Linnemann 1941 var. *ramanniana*), a zygomycete with a low growth rate (the colony diameter not exceeding 40 mm after 5 days of cultivation on wort agar at 25°C);

(4) VKM F-663—*Cunninghamella echinulata* (Thaxter 1891) Thaxter 1905, a rapidly growing strain (the colony diameter up to 70 mm after 4 days of cultivation on wort agar at 25°C).

Fungal spores. The cultures were grown on agarized wort (7°B) for 12 days at 28°C. The spores were washed off with 150 mL of sterile distilled water, filtered through a nylon filter to remove mycelial fragments, and centrifuged for 10 min at 3500 rpm.

The spores were then washed with 20 mL of deionized water and centrifuges; this procedure was repeated twice. A fraction of the spore suspension (spores 0) was used for sample preparation immediately after washing, while another fraction (spores G) was centrifuged, transferred into sterile distilled water, and incubated at 28°C on a shaker (130 rpm) to achieve germination. When the shares of swollen and germinating spores (with formed growth tubes) approached 100 and 60%, respectively, the suspension was centrifuged for 10 min at 3500 rpm. Washed spores were resuspended in 20 mL deionized water, and spore suspension (5 μL) was applied to 3-mm copper grids with carbonized formvar coating. The samples were air-dried for 24 h at room temperature and carbon-sprayed at 90°.

Electron microscopy and X-ray microanalysis were carried out using a JEM-1400 electron microscope (Jeol, Japan) equipped with X-ray microanalysis system Aztec TEM advanced with X-Max 80T detector (Oxford Instruments, United Kingdom) at 80 keV and the sample tilting angle of 15°. The spectra were analyzed using the AZtec program (Oxford Instruments, United Kingdom).

Lipid extraction from dry spore biomass was carried out according to Folch et al. (1957). The lipids were quantified by gravimetric analysis.

Trehalose extraction and quantification was carried out using HPLC as described previously (Mysyakina et al., 2016b).

Statistical analysis was carried out using Microsoft® Office Excel® 2007. The experiments were conducted in five to eight replicates.

RESULTS AND DISCUSSION

X-ray microanalysis provides for rapid determination of the elemental composition of microbial objects (both individual cells and cell aggregates), thus making it possible to characterize the state of a spore population as a whole. Since the spore samples were mounted on carbon-reinforced formvar films, carbon content was determined both for the areas containing spores and for the spore-free (background) ones, and carbon content was calculated as the difference between these values (see example on Fig. 1).

Images of dormant and germinating *A. tamarii* VKM F-64 conidia and *C. echinulata* VKM F-663 sporangiospores used for determination of elemental composition are presented on Figs. 2 and 3, respectively. The swollen and germinating spores G of rapidly germinating strains (*C. echinulata* VKM F-663 and *A. tamarii* VKM F-64) were used for analysis after 5-h incubation in sterile distilled water. In the case of slowly germinating strains (*A. sydowii* VKM F-441 and *U. ramanniana* VKM F-582) the incubation time was 20 h.

The following elements were of special interest: carbon, the major biogenic element; calcium as a secondary cell effector and a stabilizer of membranes and macromolecules; potassium, which is involved in development of the transmembrane potential, water metabolism, and maintaining of osmotic pressure; sulfur, a component of proteins, and phosphorus, a component of nucleotides (including ATP), nucleic acids, phospholipids, and energy equivalents. We expected the K/Ca and P/S ratios to reflect the properties of elemental composition in the dormant and germinating spores of mycelial fungi with different rates of transition from the dormant state in the absence of nutrients in the incubation medium.

Apart from these elements, the spores contained oxygen (1.5 to 10.4%), magnesium (0.2 to 0.9%), silicon (0.2 to 0.4%), chlorine (0.2 to 0.5%), iron (0.1 to 0.6%), arsenic (0.1 to 1.0%), and other minor elements.

Concentrations of carbon, phosphorus, sulfur, potassium, and calcium in the dormant and germinating spores of ascomycete and zygomycete fungi are presented in Table 1.

The relative content of carbon was higher in the dormant spores of slowly germinating fungi *A. sydowii* and *U. ramanniana* than in the spores of rapidly germinating *A. tamarii* and *C. echinulata* (Table 1). High carbon content in the spores evidently correlated with

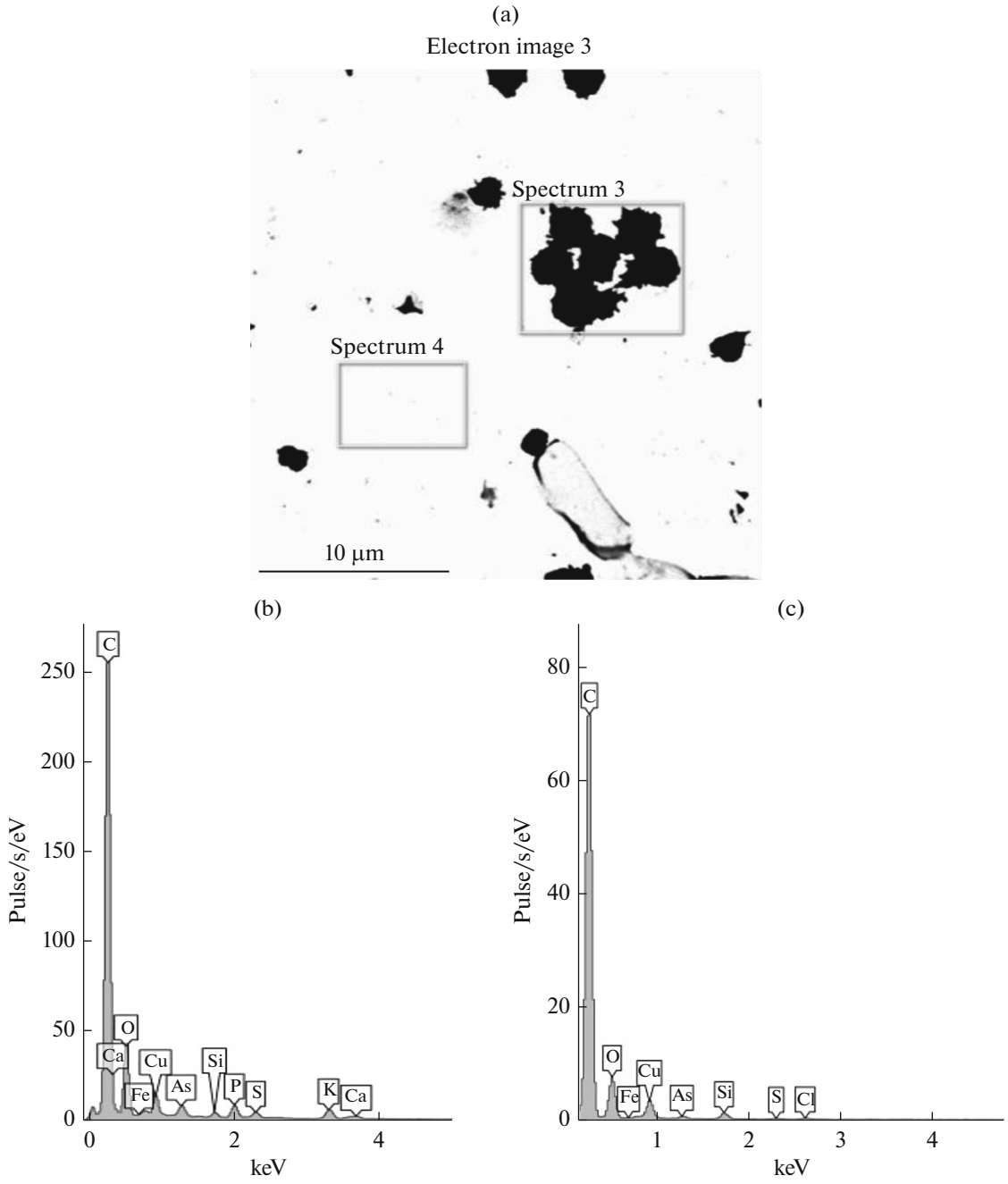

Fig. 1. Electron microscopic image of *Aspergillus sydowii* VKM F-441 spore suspension (a) and element concentrations in the areas of spore aggregation (b, spectrum 3) and backgorund (c, spectrum 4).

the levels of the major storage compounds (especially lipids), which are typical of the dormant cells and are required as energy sources for germination. The relative lipid content was higher in the spores of slowly germinating strains: 30–40% of dry biomass for *A. sydowii* and *U. ramanniana* and 10–20% in rapidly germinating spores of *A. tamarii* and *C. echinulata*. Contrarywise, the content of trehalose, the major dormancy carbohydrate with a function of a chemical chaperon, was higher in the rapidly germinating spores of *A. tamarii* and *C. echinulata* (up to 5.62 and 8.87% of the dry biomass, respectively), compared to 2.47 and 0.34% for the slowly germinating spores of *A. sydowii* and *U. ramanniana*, respectively (Mysyakina et al., 2016b). Thus, a correlation was observed between trehalose concentration and germination rate.

We have previously shown spore germination to be accompanied by changes in the composition of storage and membrane lipids, as well as of fatty acids (Mysya-

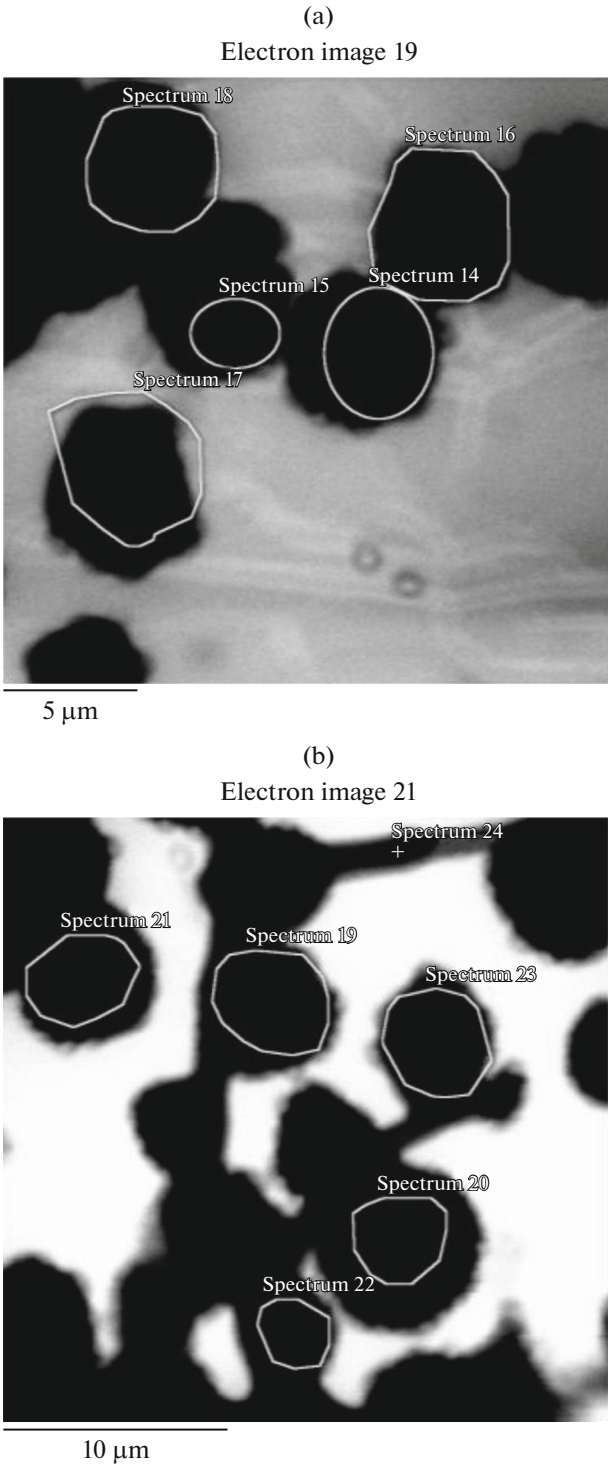

Fig. 2. Dormant (a) and germinating (b) spores of *Aspergillus tamarii* BKM F-64.

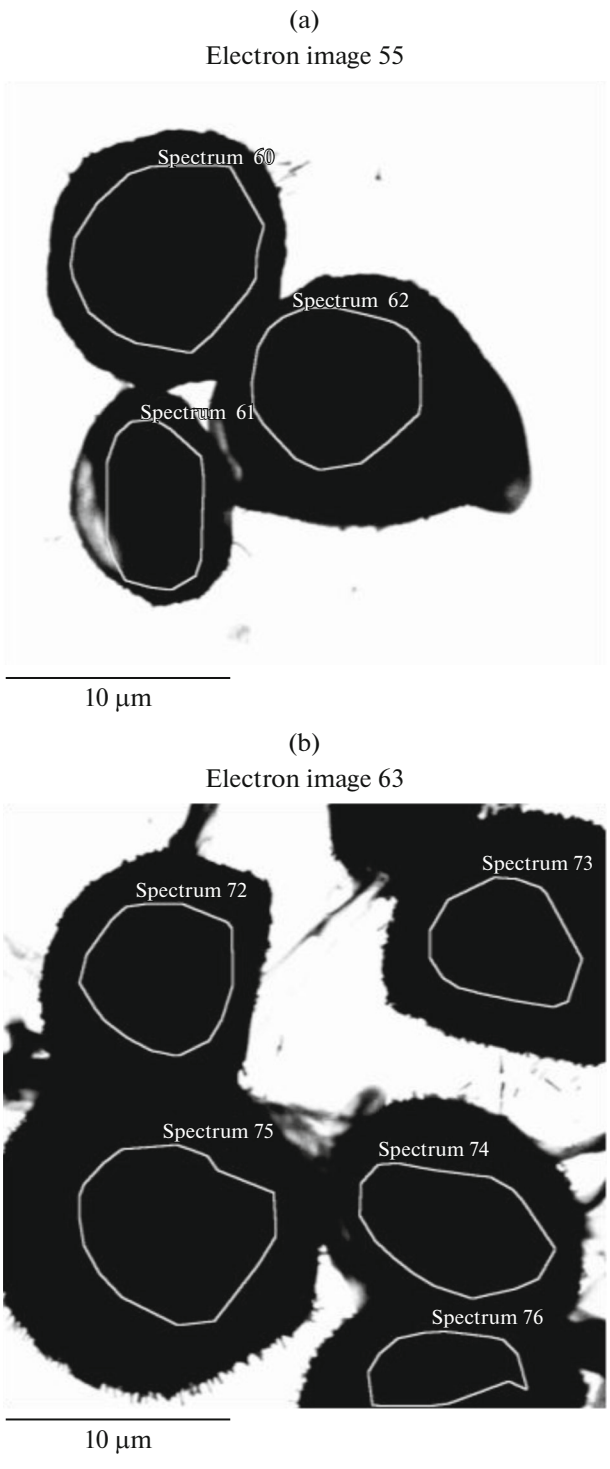

Fig. 3. Dormant (a) and germinating (b) spores of *Cunninghamella echinulata* VKM F-663.

kina et al., 2018). Thus, the relative content of phosphatidylcholine (PC), one of the massive membrane phospholipids, decreased in the spores of *C. echinulata*, *U. ramanniana*, and *A. sydowii*. Interaction of various ions with the membrane structures in living objects and in model systems has been described in the literature (Hodgkin and Horowicz, 1959; Träuble and Eibl, 1974; Song et al., 2014; Friedman, 2018). Polar groups of the lipid membranes were shown to interact directly with ions, which may affect the membrane

Table 1. Contents of carbon, phosphorus, sulfur, potassium, calcium, and magnesium in dormant (spores 0) and germinating (spores G) spores of ascomycete and zygomycete fungi with different germination rates

Strain	Spores 0						Spores G					
	elements, % of the total											
	C	P	S	K	Ca	Mg	C	P	S	K	Ca	Mg
A. sydowii VKM F-441	78.12 ± 0.32	0.72 ± 0.4	0.24 ± 0.05	0.62 ± 0.2	0.30 ± 0.15	Tr.*	70.02 ± 0.15	0.1 ± 0.01	0.17 ± 0.01	0.07 ± 0.01	Tr.*	Tr.*
A. tamarii VKM F-64	49.62 ± 2.8	1.76 ± 0.21	0.88 ± 0.11	1.15 ± 0.17	0.66 ± 0.09	0.5 ± 0.07	60.18 ± 2.99	2.67 ± 0.25	1.25 ± 0.1	2.03 ± 0.34	0.69 ± 0.06	0.64 ± 0.03
U. ramanniana VKM F-582	52.64 ± 10.09	6.01 ± 1.83	0.67 ± 0.16	2.19 ± 0.52	0.89 ± 0.31	0.58 ± 0.19	40.19 ± 8.01	3.64 ± 1.7	0.85 ± 0.18	1.09 ± 0.44	0.57 ± 0.24	0.33 ± 0.15
C. echinulata VKM F-663	37.28 ± 4.87	1.84 ± 0.45	0.42 ± 0.11	1.42 ± 0.52	1.75 ± 1.24	0.24 ± 0.05	37.74 ± 7.71	2.95 ± 0.57	0.57 ± 0.13	2.21 ± 0.52	3.13 ± 0.61	0.23 ± 0.04

* Below detection limit.

Table 2. The P/S and K/Ca ratios in dormant (spores 0) and germinating (spores G) spores of ascomycete and zygomycete fungi with different germination rates

Strain	Element ratios			
	P/S		K/Ca	
	spores 0	spores G	spores 0	spores G
A. sydowii VKM F-441	3.06	0.59	2.08	– *
A. tamarii VKM F-64	2.0	2.14	1.74	2.94
U. ramanniana VKM F-582	8.97	4.28	2.46	1.91
C. echinulata VKM F-663	5.12	5.22	0.91	0.75

* Ca content was below detection limit.

properties, including phase transition of the lipids (Träuble and Eibl, 1974), membrane potential (Hodgkin and Horowicz, 1959), and hydration layer dynamics (Song et al., 2014). Specific interactions depend also on the lipid composition of the membranes. Among the lipids forming biological membranes, many are zwitterionic (PC, PEA) or charged (PS, CL). Na^+, K^+, and Cl^- ions are common in biologically important electrolytes, while bivalent cations Ca^{2+} and Mg^{2+} may catalyze membrane fusion (Portis et al., 1979; Wilschut et al., 1980) or modify the membrane structure by simultaneous binding to several anionic sites.

In our opinion, comparison of pairwise ratios of certain elements may provide more information than comparison of their individual levels. While the P/S ratio was lower in *Aspergillus* dormant spores than in the spores of zygomycetes (Table 2), this ratio in rapidly germinating spores of *A. tamarii* and *C. echinulata* was 1.5–1.75 times lower than in slowly germinating spores of *A. sydowii* and *U. ramanniana*. Thus, in the case of relatively low P/S ratio in the spores, more rapid transition from the dormant state may be expected. Importantly, the P/S ratio of rapidly germinating spores of *A. tamarii* and *C. echinulata* changed insignificantly in the course of germination, while in the case of slowly germinating *A. sydowii* and *U. ramanniana* it decreased two- and fivefold, respectively (Table 2).

The K/Ca ratio in the dormant spores 0 of *A. tamarii* and *C. echinulata* was also lower than in spores 0 of *A. sydowii* and *U. ramanniana*. While no clear pattern of K/Ca changes in the course of germination of *A. tamarii* conidia was found, slowly germinating spores of *A. sydowii* and *U. ramanniana* strains exhibited a decrease in the K/Ca ration during transition from the dormant state. This issue probably requires further research. Calcium is known to play an important part in the regulation of signal transduction, spore germination, growth, and morphogenesis; it may be toxic to the cells, and its concentration depends on a number of transporters and on expression of the genes responsible for calcium homeostasis (Warwar and Dickman, 1996; Prithviraj et al., 1998; Osherov and May, 2001; Pittman, 2011; Dinamarco et al., 2012).

Investigation of the elemental composition of germinating fungi, as well as of the membrane–ion interactions in germinating fungal spores, is important for

the understanding of effect of ions on the structure of two-layered membranes and thus on the interaction of the membranes with other molecules, such as proteins, including enzymes, and other compounds synthesized (mainly from endogenous resources) in the course of spore transition from the state of exogenous dormancy.

Mycelial fungi are among the major organisms used for biological synthesis, including the poorly studied zygomycete species *U. ramanniana*, one of which strains was studied in the present work. It is considered promising for biochemical and biotechnological applications due to its tolerance to benomyl fungicides and the presence of oleogenic properties. Expression of the *U. ramanniana* diacylglycerol O-acyltransferase 2A (DGAT2A) gene in soy seeds resulted in increased oil production and did not affect other important parameters. The estimated yearly profit from this increased oil production may exceed US$ 1 billion (Lardizabal et al., 2008; Grigoriev et al., 2014).

The results of the present work, as well as our previous results (Mysyakina et al., 2014), indicate importance of investigation of the elemental composition of dormant and germinating fungal spores, including their P/S ratio, for assessment of their physiological state and as an indicator of potential rate of emergence from the dormant state, which is essential for biotechnological applications of mycelial fungi.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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