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Morphological Characteristics of Natural Strains of Certain Species of Basidiomycetes and Biological Analysis of Antimicrobial Activity under Submerged Cultural Conditions

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Abstract—Twenty-one strains belonging to 18 species of basidiomycetes from different ecological groups of fungi were isolated from natural sources. Light and electron microscopy was used to determine the morphological properties of the cultures, which confirmed their classification as basidiomycetes and facilitated their identification in monocultures. The capacity of the fungal strains for biosynthesis of antibiotics was determined by one- or two-stage cultivation on seven nutrient media. It was established that, under submerged cultivation, antimicrobial substances were formed by 13 strains (81.25%) of 12 fungal species (*Armillaria* sp., *Coprinus comatus, Flammulina velutipes, Hypsizygus ulmarius, Lentinus tigrinus, Lycoperdon pyriforme, Macrolepiota procera, Panellus serotinus, Pholiota aurivella, Pholiota lenta, Rhodocollybia maculate*, and *Sparassis crispa*). The antibiotics formed were efficacious against bacterial test strains, including the methicillin-resistant strain *Staphylococcus aureus* (MRSA) and the strain *Leuconostoc mesenteroides* VKPM B-4177 that is resistant to the glycopeptide antibiotics. No antibiotic activity was revealed against fungal test cultures (*Aspergillus niger* INA 00760 and *Saccharomyces cerevisiae* RIA 259).

Keywords: basidiomycetes, morphological characteristics, antimicrobial substances.
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Over the last decades, increasing attention has been given to diverse compounds obtained from higher basidial fungi. Compounds of fungal origin are used in pharmacology [1–4], the food and textile industries [5], purification of sewage and removal of contaminants from natural ecosystems [6, 7], manufacture of hygienic and cosmetic items, etc. Presently, however, only a small part of the promising fungal species have been investigated. The lack of knowledge of the biology of many representatives of this heterogeneous group is one of the factors containing the development of this kind of works.

Due to the propagation of drug-resistant pathogenic bacteria and, as a consequence, a decrease in the number of efficacious antimicrobial medications, the need for investigating new antibiotics is increasing [8–10]. One of the approaches to investigating new antibiotics is to study the species of the assumed producers of new antibiotics that have been previously not studied or poorly studied.

Since the discovery of penicillin in 1928, fungi have been the main object of search for new antibiotics. At present, antibiotics have been reported in a wide range of prokaryotes and eukaryotes. It should be noted that, in recent years, fungi, including higher ones, have again become the most numerous group of organisms in this field of investigations [11, 12]. One of the requirements for such potential producers is cultivation under submerged conditions, which makes it possible, upon finding a promising compound, to develop the technology and to produce it by means of biosynthesis.

The goal of the present work was to study the morphology of the strains of certain species of basidiomycetes isolated from natural habitats and carry out biological analysis of their antimicrobial activity in submerged culture.

MATERIALS AND METHODS

The macromycete fruit bodies were collected from June to October 2006 and 2007 in Moscow and Moscow oblast (the vicinity of the Skadovskii Moscow University Biological Station, Zvenigorod), in Rostov oblast, and in Krasnodar krai (the vicinity of Sochi). Pure cultures were obtained by the tissue method.

The cultures (21 strains, 18 species) of different taxonomic groups were used in this work. Agaricoid basidiomycetes of different families (*Agaricaceae, Marasmiaceae, Mycenaceae, Paxillaceae*, *Polypora-*

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Table 1. Species identity, the region of sampling, and the dates of isolation of pure cultures

Species	Number of strains	Isolation date of pure culture	Place of collection of fruit bodies
Armillaria sp. 3920	1	October 2006	Rostov oblast
Clitocybe nebularis (Batsch) P. Kumm. 3921	1	September 2007	Moscow oblast
Coprinus comatus (O.F. Müll.) Pers. 3922	1	October 2007	Moscow
Flammulina velutipes (Curtis) Singer 3923	1	October 2007	Korolev
Hypsizygus ulmarius (Bull.) Redhead 3925	1	September 2006	Moscow oblast
Kuehneromyces mutabilis (Schaeff.) Singer & A.H. Sm. 3926	1	October 2007	Moscow oblast
Lentinus tigrinus (Bull.) Fr. 3927	1	October 2006	Rostov oblast
Lepista irina (Fr.) H.E. Bigelow 3928	1	October 2006	Rostov oblast
Lycoperdon perlatum Pers. L-1	1	July 2007	Moscow oblast
Lycoperdon pyriforme Schaeff. 3929	1	September 2007	Moscow oblast
Macrolepiota procera (Scop.) Singer 3930	1	September 2006	Moscow oblast
Panellus serotinus (Schrad.) Kühner 3931, 3932	2	October 2007	Moscow oblast
Paxillus involutus (Batsch) Fr. P-1	1	September 2007	Moscow oblast
Pholiota aurivella (Batsch) P. Kumm. 3933	1	October 2006	Rostov oblast
Pholiota lenta (Pers.) Singer 3934	1	October 2006	Moscow oblast
Pholiota squarrosa (Batsch) P. Kumm. 3935–3937	3	September 2006, september 2007	Moscow, Losiny Ostrov, Moscow oblast
Rhodocollybia maculata (Alb. & Schwein.) Singer 3938	1	September 2007	Moscow oblast
Sparassis crispa (Wulfen) Fr. 3939	1	September 2007	Moscow

ceae, *Strophariaceae, Tricholomataceae*) constituted most of the cultures. The cultures of aphyllophoroid (*Sparassis crispa*) and gasteroid basidiomycetes (*Lycoperdon perlatum* and *L. pyriforme*) were also included in the studies. The main attention was given to xylotrophic fungi, since biologically active substances were revealed in many representatives of this ecologotrophic group and this group is considered to be the most promising for searching for their producers. The litter saprotrophs were widely represented as well. In addition, humus saprotrophs (the industrially cultivated *Coprinus comatus*) were included in the work. For greater coverage of the diversity of macromycetes, parasitic species (*Armillaria* sp.) and the species developing on buried wood (*Sparassis crispa*), as well as the mycorrhiza-forming *Paxillus involutus*, were included in the group of xylotrophic fungi. The species used in this work are presented in Table 1.

Agar media (modified Czapek medium with crystallized cellulose as a source of carbon, agar wheat decoction) and wood materials (sterilized wooden cubes moistened with wheat decoction) were used for culture storage. Wood (asp, alder, birch, or oak) was selected taking into account the kind of the tree from which the strain was isolated.

For surface cultivation of the fungi, media conventionally used for wide species and trophic spectrum of macromycetes were used: agarized wort (4°B, 2% agar), malt agar, potato–glucose agar, and agarized wheat agar [13–15]. After preliminary experiments, we found it necessary to double the concentrations of the potato–glucose and wheat media.

The agar media were inoculated with agar slabs at the center of a petri dish in three repeats. The cultures were incubated at 24°C.

The linear growth rate on agarized beer wort was measured in the course of the experiments.

The basidiomycetous mycelium growing on agarized beer wort (the 7th–18th day of growth) was examined using a light microscope (Axioskop 40FL) and a scanning electron microscope (JSM-6380). The number of nuclei in the mycelial cells (staining with DAPI [16]) and spores; the presence of clamp connections and the regularity of their distribution; the anamorph type; the presence of secretory cells and crystal-incrusted hyphae; the formation of vesicular cells, their location and structure; the presence of anasto-

moses, cords, and other hyphal structures were noted in the process of study.

The objects for study of antimicrobial activity were the following 16 strains of 13 species of basidial fungi: *Armillaria* sp. 3920; *Coprinus comatus* 3922; *Flammulina velutipes* 3923; *Hypsizygus ulmarius* 3925; *Lentinus tigrinus* 3927; *Lycoperdon pyriforme* 3929; *Macrolepiota procera* 3930; *Panellus serotinus* 3931 and 3932; *Pholiota aurivell*a 3933*; Pholiota lenta* 3934; *Pholiota squarrosa* 3935, 3936, and 3937; *Rhodocollybia maculate* 3938; and *Sparassis crispa* 3939. The macromycetes not included in the analysis demonstrated poor mycelial growth under these conditions in liquid laboratory media.

The following 12 test cultures were used for determining the antimicrobial activity: gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *B. mycoides* 537, *B. pumilis* NCTC 8241, *Leuconostoc mesenteroides* VKPM B-4177, *Micrococcus luteus* NCTC 8340, *Staphylococcus aureus* FDA 209P (MSSA), INA 00761 (MRSA, clinical isolate), gram-negative bacteria (*Escherichia coli* ATCC 25922, *Comamonas terrigena* VKPM B-7571 (=ATCC 8461), *Pseudomonas aeruginosa* ATCC 27853), and fungi (*Aspergillus niger* INA 00760, *Saccharomyces cerevisiae* RIA 259).

For the test cultures, modified Gauze medium no. 2 was used containing the following (%): glucose, 1; peptone, 0.5; tryptone, 0.3; NaCl, 0.5; and agar, 2.

Media for submerged cultivation (%). Medium P-1: glucose, 1.0; soybean flour, 2.0; Maltax malt extract (Finland), 2.0. Medium P-2: glucose, 1.0; soybean flour, 2.0; Maltax malt extract (Finland), 2.0; distillery dregs, 25.0. Medium M-1: Maltax malt extract (Finland), 1.0; distillery dregs, 50.0. Medium M-2: Maltax malt extract (Finland), 2.0. Medium M-4: Maltax malt extract (Finland), 4.0. Medium L-2: Lager malt extract (Finland), 2.0. Medium 2663: glycerol, 3.0; soybean flour, 1.5; NaCl, 0.3; chalk, 0.3. Medium 5339: glycerol, 2.0; soybean flour, 0.5; $(NH_4)_2SO_4$, 0.15; NaCl, 0.3; chalk, 0.3.

The basidial fungi, as well as the test cultures *L. mesenteroides* VKPM B-4177, *C. terrigena* VKPM B-7571 (=ATCC 8461), *A. niger* INA 00760, and *S. cerevisiae* RIA 259, were incubated at 28°C. The remaining test cultures were incubated at 37°C. The duration of cultivation of the test cultures was 17–20 h.

Submerged cultivation of basidial fungi was carried out in 750-ml Erlenmeyer flasks with 150 ml of the medium on a rotor shaker (200 rpm). For one-stage cultivation, the flasks were inoculated with the culture from the agar medium. In two-stage cultivation, the submerged culture grown in the first medium was introduced into the second medium in an amount of 5% (vol/vol). The activity in the culture liquid was determined once a week on the 7th, 14th, 21st, and 28th days of growth.

The antimicrobial activity in the culture liquid was determined using the agar diffusion method. The test cultures (10^7 cells/ml) were introduced into cooled, melted modified Gauze medium no. 2. Upon solidification of the agar medium, 100 μl of the culture liquid was introduced into each 9-mm well.

In order to concentrate the antimicrobial substances, ethyl acetate (the volume ratio 2 : 1) was added to the culture liquid; the mixture was shaken vigorously and allowed to stand at 4°C for 20 h. The ethyl acetate layer was removed and evaporated, and the precipitate was diluted in 10% methanol. Solution of the concentrate was applied to the filter paper disks (6 mm in diameter); the disks were air-dried and placed on the surface of the medium with the test cultures. Considering the amount of the concentrate applied to the disk, the sample analyzed contained approximately 15 times more substances than did 100 μl of the culture liquid introduced into a well.

The antimicrobial activity was determined by the diameters of the test culture growth delay zones after incubation.

RESULTS AND DISCUSSION

The cultures of the species studied were clearly divided into three groups according to vegetative growth rate (Table 2). On the seventh day of growth, the radii of the colonies of most of the species were 1–5 cm. *Armillaria* sp. 3920, *L. perlatum* L-1, *P. involutus* P-1, and *S. crispa* 3939 were slowly growing (<1 cm on day 7 of growth); *F. velutipes* 3923, *L. tigrinus* 3927, and *R. maculate* 3938 grew quickly (>1 cm on day 7 of growth). The latter species may be of interest from the point of view of rapid biomass accumulation, which is confirmed by a large number of biotechnological investigations with *F. velutipes* and *L. tigrinus* [17].

Data on the micromorphological characteristics of the cultures studied are shown in Table 3.

All the cultures, except for *Armillaria* sp. 3920, had binuclear mycelial cells. The cells of this species were mononuclear (Table 3, Fig. 1), which agrees with the literature data [16].

Regular clamp connections were observed in most species of the macromycetes studied. Irregular clamp connections are characteristic of the species *M. procera* 3930. They were absent in *Armillaria* sp. 3920, *C. comatus* 3922, *L. perlatum* L-1, and *L. pyriforme* 3929 (Table 3). The unusual structure of the clamp connections in *K. mutabilis* 3926, which were characterized by thickenings at the site of contacts with the hyphae (Fig. 2), should be mentioned.

In many cultures, we observed various morphological manifestations of secretory activity on the 18th day of growth: the secretory hyphae incrusted with crystals and secretory drops on the mycelium (from minute to large ones covering large areas of the hypha). The formation of secretory hyphae with crys-

Table 2. Vegetative growth rate on agarized beer wort medium

Low growth rate (<1 cm over 7 days)	Average growth rate (1–5 cm over 7 days)	High growth rate (>5 cm over 7 days)
Armillaria sp.	*Clitocybe nebularis*	*Flammulina velutipes*
Lycoperdon perlatum	*Coprinus comatus*	*Lentinus tigrinus*
Paxillus involutus	*Hypsizygus ulnarius*	*Rhodocollybia maculate*
Sparassis crispa	*Kuehneromyces mutabilis*	
	Lepista irina	
	Lycoperdon pyriforme	
	Macrolepiota procera	
	Panellus serotinus	
	Pholiota aurivella	
	Pholiota lenta	
	Pholiota squarrosa	

tals was observed in *K. mutabilis* 3926 (plate-shaped crystals), in *L. pyriforme* 3929 (rod-shaped and acicular crystals), in *M. procera* 3930 (abundant acicular crystals), in *P. aurivella* 3933 (minute rhomboid crystals), in *P. lenta* 3934 (massive, pointed crystals in fascicles), in the strains *P. squarrosa* 3935–3927 (single acicular crystals), and in *S. crispa* 3939 (acicular crystals forming fascicles and tetrahedral crystals; Fig. 3a). Teardrop secretory discharge on the hyphal surface was observed in *L. irina* 3928 (Fig. 3b), *S. crispa* 3939, and *M. procera* 3930 (Fig. 3c).

In *Armillaria* sp. 3920, we observed the formation of rounded swollen cells with large vacuoles (vesicular cells) along with the usual mycelium cells (Fig. 4a). The apical mycelial cells of *C. comatus* 3922 were swollen at the hyphal tip (Fig. 4b).

In two basidiomycete species, *L. pyriforme* 3929 and *S. crispa* 3939, the formation of mycelial cords (synnema) was observed. The cords were very robust and consisted of ten or more closely located and anastomosing hyphae (Figs. 5a, 5b, respectively).

As early as day 10, the mycelium of *L. tigrinus* 3927 formed a thick mycelial mat consisting of fine sclerotized hyphae with thick cell walls (resembling dichofibres [18]), and on the 18th day of growth the formation of primordia was observed.

In a number of species, the formation of mitotic spores (arthrospores) was observed. Arthrospore formation was observed in the cultures of *F. velutipes* 3923 (abundant) as described earlier [19, 20], *H. ulmarius* 3925 (in chains) as described earlier [21], *P. aurivella* 3933 (sympodially branching conidiophores) as

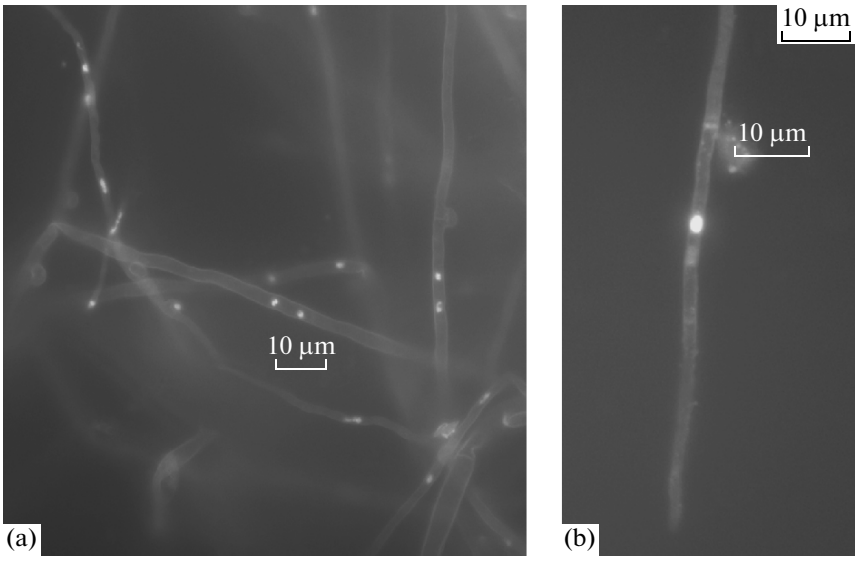

Fig. 1. Binuclear cells of *Lepista irina* 3928 (a) and the mononuclear cells of *Armillaria* sp. 3920 mycelium (b).

Table 3. Micromorphology* of the basidiomycete monoculture mycelium

Species	Number of nuclei in mycelial cells/spores	Clamp connections	Arthros pores	Secretory cells	Vesicular cells	Cords	Crystals
Armillaria sp. 3920	1	–	–	–	+	–	–
Clitocybe nebularis 3921	2	+	–	–	–	–	–
Corpinus comatus 3922	2	–	–	–	–	–	–
Flammulina velutipes 3923	2/1	+	+	–	–	–	–
Hypsizygus ulmarius 3925	2/1–4	+	+	–	–	–	–
Kuehneromyces mutabilis 3926	2	+	–	–	–	–	+
*Lentinus tigrinus*** 3927	2	+	+	–	–	–	–
Lepista irina 3928	2	+	–	+	–	–	–
Lycoperdon perlatum L-1	2	–	–	–	–	–	–
L. pyriforme 3929	2	–	–	–	–	+	+
*Macrolepiota procera**** 3930	2	+ not uniformly	–	+	–	–	+
Panellus serotinus (two strains) 3931, 3932	2	+	–	–	–	–	+
Pholiota aurivella 3933	2/2	+	+	–	–	–	–
P. lenta 3934	2/2	+	+	–	–	–	+
P. squarrosa 3935–3937	2	+	+/– ****	+/–	–	–	+/–
Rhodocollybia maculata 3938	2	+	–	–	–	–	–
Sparassis crispa 3939	2	+	–	+	–	+	+

Notes: * Anastomoses were present in all the cultures.

** Solid mycelial dense and pigmented mat in the form of a crust with primordia, consisting of the usual mycelium with clamp connections and binuclear cells and sclerotized, abundantly branching fine nuclear-free hyphae (dichofibers according to Clémençon, 2004).

*** Strong smell of mushrooms.

**** The structures were not observed in all the strains studied.

described earlier [19, 20], and *P. lenta* 3934 (sympodially branching conidiophores [20] (Fig. 6), in the strains *P. squarrosa* 3935–3937 (single, apical), and in *L. tigrinus* 3927 (single, intercalary). The mycelium of *F. velutipes* 3923 actually disintegrated into mononuclear arthrospores, which were suitable for obtaining homokaryotic isolates. In the representatives of the genus *Pholiota*, the arthrospores contained two nuclei, while in *H. ulmarius* 3925 the number of nuclei in the arthrospores varied between 1 and 4 (Fig. 7).

Thus, of the 20 strains identified with 18 species of basidiomycetes, only 2 strains (*C. nebularis* 3921, *R. maculate* 3938) did not reveal any morphological peculiarities upon cultivation on agarized beer wort medium. Under these conditions, these strains formed a mycelium consisting of binuclear cells with clamp connections. The remaining cultures had a number of micromorphological features that made it possible to identify them in a monoculture and assign them to basidiomycetes due to the presence of clamp connections (except for *Armillaria* sp. 3920, *Lycoperdon perlatum* L-1, and *L. pyriforme* 3929).

In order to reveal antimicrobial activity, the fungi were cultivated in different nutrient media. At first, one-stage cultivation in the above-mentioned media was carried out, and, in a number of cases, two-stage cultivation was carried out in order to increase the level of biosynthesis of antimicrobial substances (Table 4).

It follows from Table 4 that, during two-stage cultivation on media P-1 and M-4, *Armillaria* sp. was active only against two *Staphylococcus aureus* strains; its activity peaked at the 14th day of growth after transfer to medium M-4 (the antibiotic component 1). Upon two-stage cultivation on media P-2 and P-1 or P-2 and M-1, the activity at the second stage of cultivation was revealed on the 14th day of growth only against the test bacterium *L. mesenteroides* BKPM B-4177 (the antibiotic component 2). Upon two-stage cultivation on media P-2 and M-1, the activity against

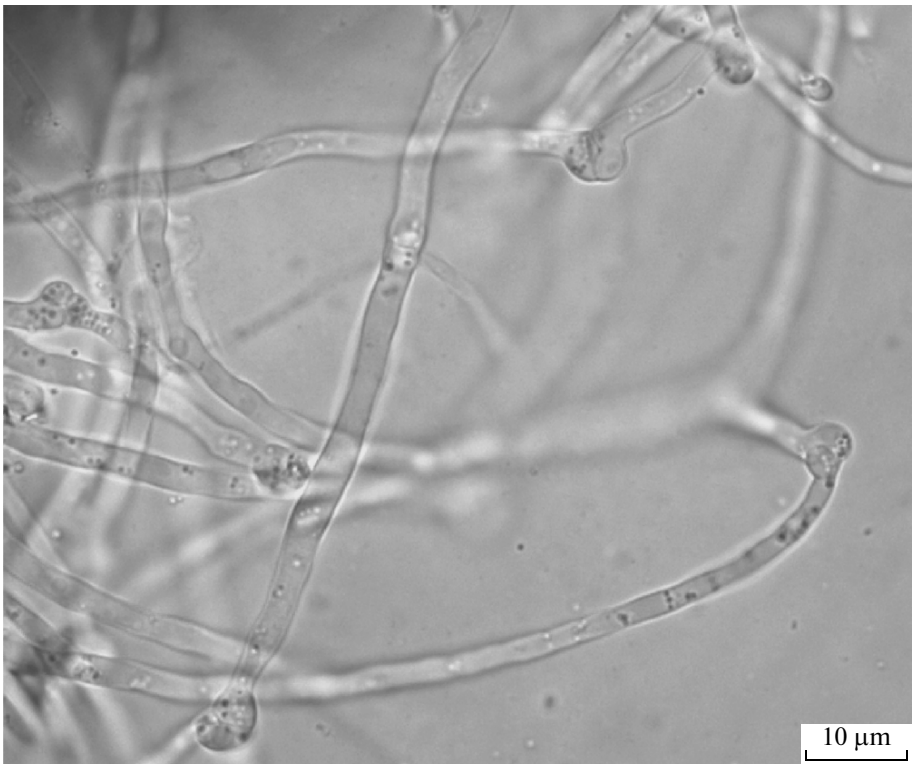

Fig. 2. Unusual morphology of the clamp connections with thickenings at the site of contacts with the hyphae in *Kuehneromyces mutabilis* 3926.

L. mesenteroides BKPM B-4177 was lost on the 21st day of growth; however, the activity against *B. subtilis* ATCC 6633 was found (the antibiotic component 3). The growth inhibition zones of *B. subtilis* ATCC 6633 were larger than those of this test organism upon one-stage cultivation on medium P-1; however, the activity against *Staphylococcus aureus* strains was absent, in contrast to one-stage cultivation. Hence, during one-stage cultivation on medium P-1, either a different substance (the antibiotic component 4) was formed or two components were formed (the antibiotic components 1 and 3).

In the *C. comatus* strain, the antimicrobial activity was only revealed upon two-stage cultivation on media P-1 and M-4 on the 21st day of growth against two gram-positive bacteria: *B. subtilis* ATCC 6633 and *S. aureus* FDA 209P (MSSA). Earlier, we showed that another strain of this species (2957) also revealed activity against MRSA and fungal cultures [22]; therefore, it may be suggested that *C. comatus* forms several antimicrobial substances with a different antimicrobial spectrum.

The *F. velutipes* strain revealed antimicrobial activity against a wide spectrum of microorganisms. An activity against *B. subtilis* ATCC 6633 was revealed upon two-stage cultivation on media P-2 and M-2 (the antibiotic component 1). Upon two-stage cultivation on P-2, activity was only revealed against *A. niger* INA 00760 (the antibiotic component 2). Upon one-stage cultivation on P-2, activity was revealed against all the five microorganisms mentioned above. Since on the 14th day of cultivation on medium P-2 at the second stage the level of biosynthesis of the component efficacious against *A. niger* INA 00760 corresponded to the level of biosynthesis upon one-stage cultivation on P-2, it may be suggested that a different compound (the antibiotic component 3) is efficacious against *S. aureus* FDA 209P, *S. aureus* INA 00760, and *E. coli* ATCC 25922. The biosynthesis of the components 1 and 3 was probably delayed during two-stage cultivation on medium P-2, in contrast to one-stage cultivation on the same medium, and, supposedly, they could only be observed upon much longer cultivation. Earlier, flammulin, an antibiotic of protein nature possessing an antitumor activity [23, 24], was described in *F. velutipes*; however, it did not exhibit antibacterial activity.

The *H. ulmarius* strain exhibited activity against two strains of *Staphylococcus aureus* upon one-stage cultivation on medium P-1 on the 21st day (the antibiotic component 1). Upon two-stage cultivation on media P-1 and M-4, activity was revealed only against the methicillin-sensitive strain *S. aureus* FDA 209P (MSSA) and *B. subtilis* ATCC 6633. The level of this activity was lower than for the component 1; therefore, we may suggest that, under these conditions, the antibiotic component 2 was formed.

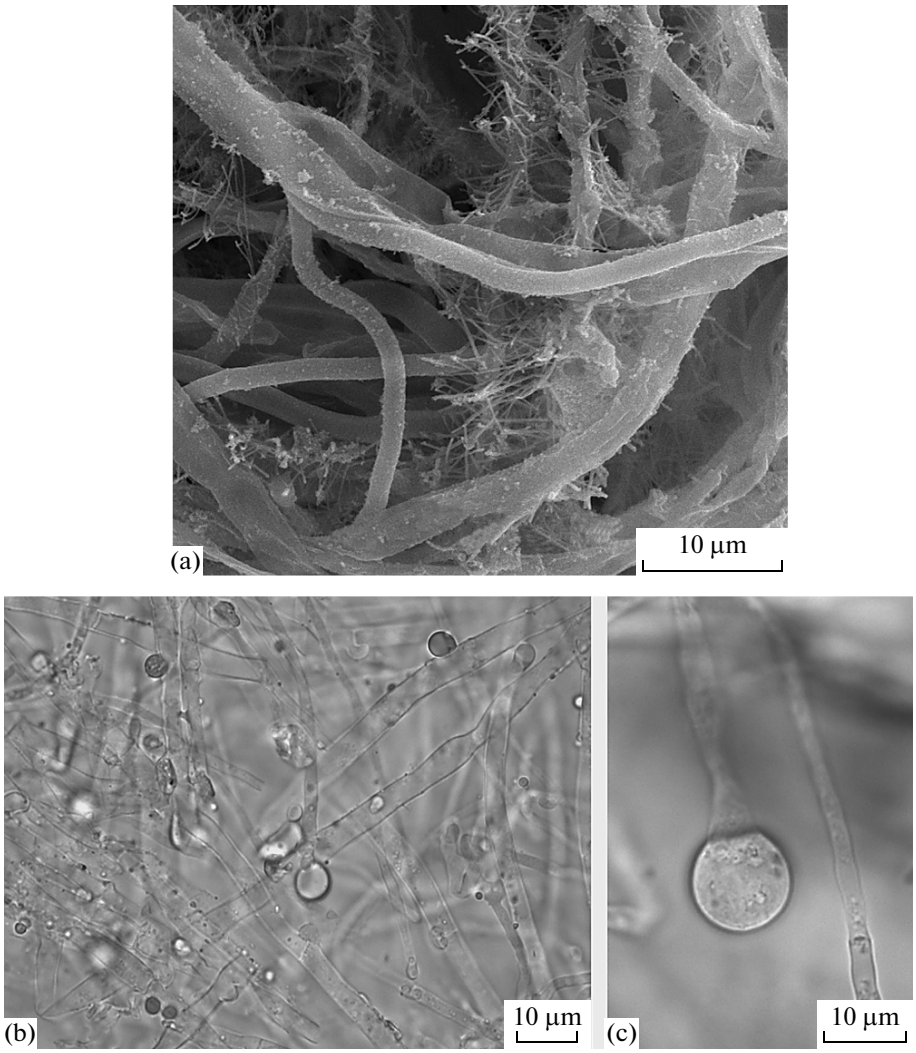

Fig. 3. Acicular crystals on the hyphae of *Lycoperdon pyriforme* 3929 (a), secretory drops on the *Lepista irina* mycelium (b), and the secretion-releasing hyphal areas in *Macrolepiota procera* 3930 (c).

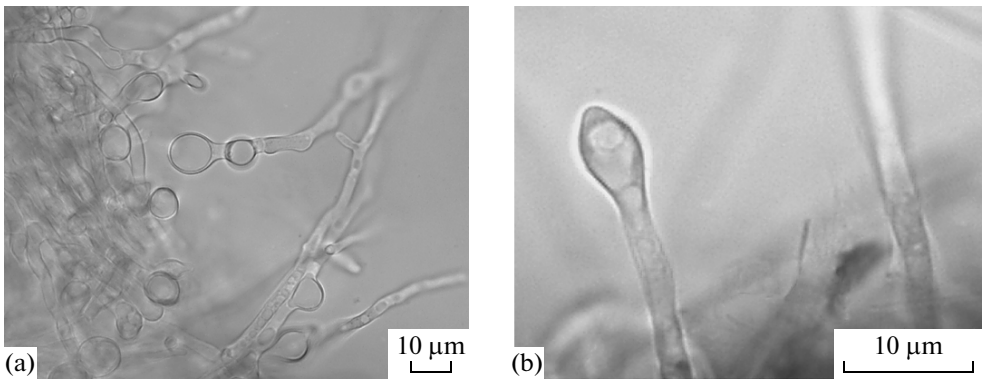

Fig. 4. Vesicular cells in *Armillaria* sp. 3920 (a) and the swollen apical mycelial cell of *Coprinus comatus* 3922 (b).

The *L. tigrinus* strain exhibited antimicrobial activity against a wide spectrum of microorganisms. The antimicrobial activity against *A. niger* INA 00760 (the antibiotic component 1) was manifested in the form of a single activity in the two cultivation variants. The highest level of antimicrobial activity against gram-

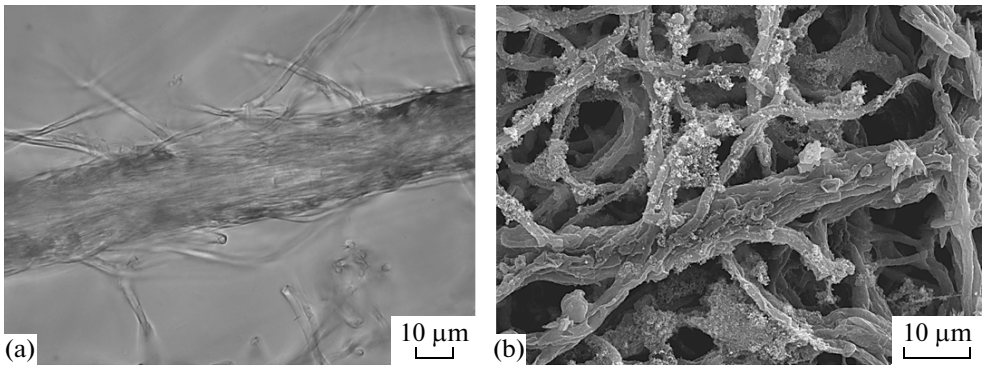

Fig. 5. Mycelial cords (sinnema) of *Lycoperdon pyriforme* 3929 (a) and *Sparassis crispa* 3939 (b).

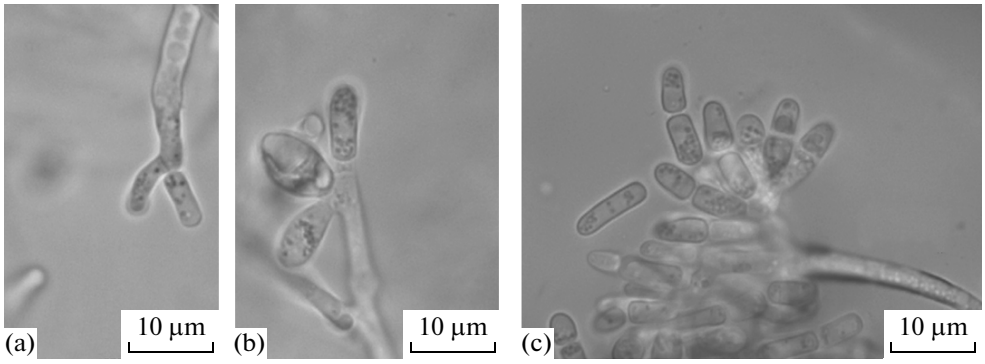

Fig. 6. Arthrospore formation in *Hypsizygus ulmarius* 3925 (a), *Pholiota aurivella* 3933 (b), and *P. lenta* 3934 (c).

positive bacteria (*B. subtilis* ATCC 6633, *S. aureus* FDA 209P) and against *E. coli* ATCC 25922 was revealed upon one-stage cultivation on medium P-1 on the 7th day of growth (the antibiotic component 2). Nevertheless, in other variants of cultivation (the highest level of biosynthesis upon two-stage cultivation on media P-1, M-1) at a lower level of activity against *B. subtilis* ATCC 6633 and *S. aureus* FDA 209P, an activity was revealed against the methicillin-resistant *S. aureus* INA 00761; therefore, we may suggest that this activity was associated with another substance (the antibiotic component 3). Upon one-stage cultivation on medium P-2, action, apart from *B. subtilis* ATCC 6633, *S. aureus* FDA 209P, and *S. aureus*

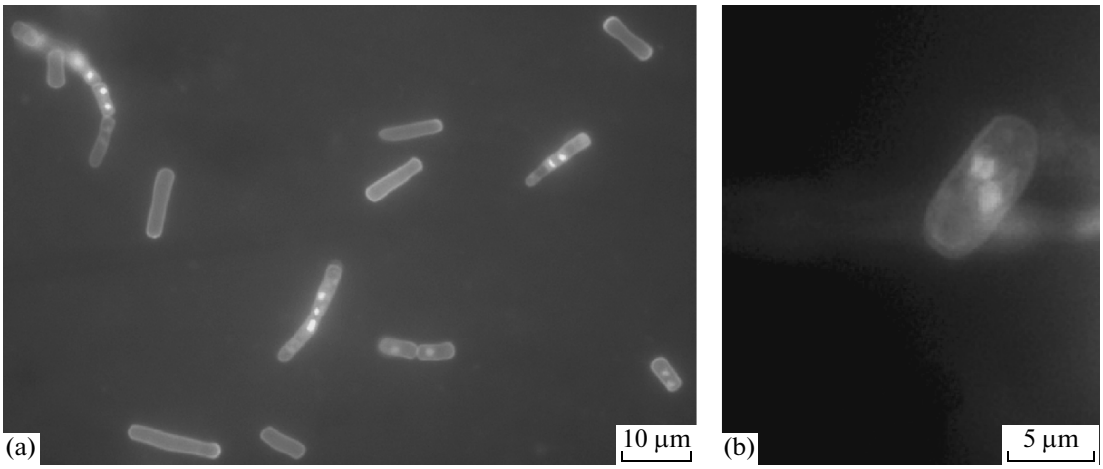

Fig. 7. Mono- and multinuclear arthrospores of *Hypsizygus ulmarius* 3925 (a) and the binuclear spores of *Pholiota aurivella* 3933 (b) (DAPI).

Table 4. Strains exhibiting antimicrobial activity under different conditions of submerged cultivation

Fungal strains	Media	Days of growth	*B. subtilis* ATCC 6633	*L. mesenteroides* VKPM B-4177	*S. aureus* FDA 209P (MSSA)	*S. aureus* INA 00761 (MRSA)	*E. coli* ATCC 25922	*A. niger* INA 00760
			Growth inhibition zone diameters (mm)					
Armillaria sp. 3920	P-1	14	10–12		10–14	10–14		
	P-1, M-4	7			10–12			
	P-2, P-1	14		16–18				
	P-2, M-1	14		10–14				
		21	12–14					
Coprinus comatus 3922	P-1, M4	21	10–12		10–14			
Flammulina velutipes 3923	P-2	21	12–16		22–25	16–18	17–19	14–18
		28	18–22		18–20	14–18	14–16	12–16
	P-2, P-2	7						10–14
		14						12–16
	P-2, M-2	14	16–20					
Hypsizygus ulmarius 3925	P-1	21			14–16	13–15		
	P-1, M-4	21	10–12		10–12			
Lentinus tigrinus 3927	P-1	7	18–22		18–20		15–18	
		14			10–12			
		21						12–14
	P-2	21	10–12	11–13	14–16	13–15		
	P-1, 2663	14			13–16			
	P-1, M-1	7	14–17		14–17	13–15		
	P-1, M-4	7	10–12		10–12			
	P-1, L-2	7			10–14	10–12		
		21						10–14
Lycoperdon pyriforme 3929	P-1	28	14–16					
	P-2	21	10–12					
		28	10–12		14–16	11–13		
	P-2	28	13–15					
	P-2, P-1	28	13–15					
	P-2, M-1	21	10–12					
Macrolepiota procera 3930	P-2, P-1	21	14–16					
	P-2, M-1	7	10–12					
Panellus serotinus 3931	P-1	7			10–14			
		14			14–17			
	P-2	21	10–12					
	P-2, P-1	7	12–14					
	P-2, M-1	7	12–14					
	P-2, M-2	21	20–24					
Panellus serotinus 3932	P-2	21	10–12		16–18			
		28			14–16			
	M-4	21			13–16			

Table 4. (Contd.)

Fungal strains	Media	Days of growth	*B. subtilis* ATCC 6633	*L. mesenteroides* VKPM B-4177	*S. aureus* FDA 209P (MSSA)	*S. aureus* INA 00761 (MRSA)	*E. coli* ATCC 25922	*A. niger* INA 00760
			Growth inhibition zone diameters (mm)					
Pholiota lenta 3934	P-2	21		10–12				18–22
		28						18–22
	M-4	21	14–18					
	P-2, P-2	7						10–12
	P-2, P-1	7						13–16
	P-2, M-1	7	10–12					15–18
Rhodocollybia maculate 3938	P-2	14	14–18					
		21		10–13	19–22			14–16
		28	17–20		23–26	20–22		13–15
	P-1	14	13–15					
		21	16–18					
	P-2, P-1	14			12–16	12–16		14–16
		21			12–14			

* No activity against *Pseudomonas aeruginosa* ATCC 27853 was revealed in any of the fungal strains.

INA 00761, was also attained against *L. mesenteroides* VKPM B-4177, which may be associated with the antibiotic component 4.

The *L. pyriforme* strain showed the peak of activity against *B. subtilis* ATCC 6633 upon one-stage cultivation on medium P-1 on day 28 (the antibiotic component 1). The strain also revealed activity against *S. aureus* FDA 209P and *S. aureus* INA 00761 on medium P-2 on the 28th day of growth; since under these conditions the level of the component 1 biosynthesis was lower than on medium P-1 on the 28th day (under the conditions when no activity against *S. aureus* strains was revealed), we may suggest that another compound (the antibiotic component 2) was active against the two strains of *S. aureus*.

The *M. procera* strain revealed activity only against *B. subtilis* ATCC 6633 in two variants of two-stage cultivation; i.e., supposedly, it formed one antibiotic.

It was shown that the *P. serotinus* strain formed at least two antibiotics of which one was efficient against *B. subtilis* ATCC 6633 (the antibiotic component 1) and the other (the antibiotic component 2) against *S. aureus* FDA 209P (MSSA). In each variant of biosynthesis, only one of the two components was synthesized. Another strain of this species (3932) was active against *S. aureus* FDA 209P (MSSA) upon one-stage cultivation on media P-2 and M-4. The activity was also revealed against *B. subtilis* ATCC 6633 at the peak of activity (one-stage cultivation on medium P-2 on day 21). Therefore, we may suggest that, under these conditions, *Panellus serotinus* 3932 formed one antibiotic substance with an antibiotic effect on *S. aureus* FDA 209P (MSSA) at a higher concentration compared to *B. subtilis* ATCC 6633.

The *P. lenta* strain showed the highest activity against *B. subtilis* ATCC 6633 upon one-stage cultivation on medium M-4 (the antibiotic component 1) and the highest activity against *A. niger* INA 00760 upon one-stage cultivation on medium P-2 on the 28th day (the antibiotic component 2). Supposedly, both antibiotic components (1 and 2) were formed upon two-stage cultivation on media P-2 and M-1. Strain *P. lenta* 3934 also revealed activity against *L. mesenteroides* VKPM B-4177 upon one-stage cultivation on medium P-1 on the 21st day of growth. The activity against *L. mesenteroides* VKPM B-4177 might be linked to the antibiotic component 3, because the efficiency against *A. niger* INA 00760 remained the same on day 28, while the efficiency against *L. mesenteroides* VKPM B-4177 disappeared.

The *R. maculate* strain showed antimicrobial activity against gram-positive bacteria and *A. niger* INA 00760. The highest activity against these strains was attained upon one-stage cultivation on medium P-2 on the 28th day of growth (the antibiotic component 1). In the other cultivation variants, with a decrease in the activity peaking against *Staphylococcus aureus* FDA 209P, the activity against the other test bacteria decreased or disappeared, but the activity against *A. niger* INA 00760, believed to be caused by another substance (the component 2), was retained.

No antimicrobial activity was revealed in the culture fluid of the *P. aurivella, P. squarrosa* (three strains), and *S. crispa* strains under the above-mentioned conditions of cultivation, in spite of satisfactory

Table 5. Summary of the results on the antimicrobial activity of the basidial fungi studied

Parameter	Strain number	%
Total number of the basidiomycete strains studied	16	100
Of them, exhibit antimicrobial activity	13	81.25
Exhibit activity against gram-positive bacteria	13	81.25
Exhibit activity against gram-positive and -negative bacteria	4	25
Exhibit activity against MRSA	7	43.75
Exhibit activity against *L. mesenteroides* VKPM B-4177	6	37.5
Exhibit activity against *L. mesenteroides* ATCC 27853	0	0
Exhibit activity against *A. niger* INA 00760	4	25

growth. Assuming that the biosynthesis of antimicrobial substances in these strains might be possible at a low level (below the sensitivity of the detection method used), we attempted to carry out their one-stage cultivation in medium M-4 with subsequent extraction with ethyl acetate and concentration. The cultivation time was extended to 35 days, and the spectrum of the test organisms used was extended to the twelve strains indicated above. As a result, an activity against gram-positive bacteria *S. aureus* FDA 209P, *P. pumilis* NCTC 8241, and *L. mesenteroides* VKPM B-4177 and the gram-negative bacterium *C. terrigena* VKPM B-7571 was established in *P. aurivella*. The highest level of activity was revealed on day 14 (against *B. pumilis* NCTC 8241 and *L. mesenteroides* VKPM B-4177) and on day 21 (against *C. terrigena* VKPM B-7571 and *S. aureus* FDA 209P), which allows us to suggest the formation of at least two antimicrobial components. The *S. crispa* strain exhibited antimicrobial activity against all the seven gram-positive test bacteria used and the gram-negative bacterium *C. terrigena* BKPM B-7571 (on days 14 and 21 at the peak level of activity on day 21); no activity against the fungal test strains was determined. Earlier, three antibiotics with antifungal activity were described in *S. crispa* [25]; therefore, the antibacterial properties of this strain were probably associated with the substances of a different nature. Of the three *P. squarrosa* strains, none exhibited antimicrobial activity. Thus, the formation of antimicrobial substances in two more of the five strains was established by means of concentration.

Table 5 shows the general conclusions drawn from the data on the antimicrobial properties of the strains studied.

It follows from Tables 4 and 5 that, under the cultivation conditions described, 81.25% of the strains studied synthesize substances inhibiting the growth of the test microorganisms. This is comparable to our previous data on the basidial fungi and to certain literature sources [26–31]. It is important to note that 43.75% of the strains produced substances that overcame the resistance of methicillin-resistant *Staphylococcus aureus* and 37.5% of the strains were efficacious against the test bacterium *L. mesenteroides* VKPM B-4177, which is characterized by a high level of resistance to glycopeptide antibiotics (500 μg/ml). Thus, the cultures studied have a high potential as producers of the antimicrobial substances overcoming two forms of drug resistance of bacteria, i.e., those forms of resistance the spread of which evokes the greatest alarm in specialists.

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