ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 1, pp. 90–98. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © A.V. Kurakov, R.B. Lavrent'ev, T.Yu. Nechitailo, P.N. Golyshin, D.G. Zvyagintsev, 2008, published in Mikrobiologiya, 2008, Vol. 77, No. 1, pp. 103–112.

EXPERIMENTAL ARTICLES

Diversity of Facultatively Anaerobic Microscopic Mycelial Fungi in Soils

A. V. Kurakov[a,1], R. B. Lavrent'ev[b], T. Yu. Nechitailo[c], P. N. Golyshin[c], and D. G. Zvyagintsev[b]

[a] *International Biotechnology Center, Moscow State University, Moscow, 119992 Russia*
[b] *Department of Soil Biology, Faculty of Soil Science, Moscow State University, Moscow, 119992 Russia*
[c] *National Biotechnology Center, Mascheroder Weg 1, 38124 Braunschweig, Germany*

Received March 26, 2007

Abstract—The numbers of microscopic fungi isolated from soil samples after anaerobic incubation varied from tens to several hundreds of CFU per one gram of soil; a total of 30 species was found. This group is composed primarily of mitotic fungi of the ascomycete affinity belonging to the orders *Hypocreales* (*Fusarium solani, F. oxysporum, Fusarium* sp., *Clonostachys grammicospora, C. rosea, Acremonium* sp., *Gliocladium penicilloides, Trichoderma aureoviride, T. harzianum, T. polysporum, T. viride, T. koningii, Lecanicillum lecanii*, and *Tolypocladium inflatum*) and *Eurotiales* (*Aspergillus terreus, A. niger*, and *Paecilomyces lilacimus*), as well as to the phylum *Zygomycota*, to the order *Mucorales* (*Actinomucor elegans, Absidia glauca, Mucor circinelloides, M. hiemalis, M. racemosus, Mucor* sp., *Rhizopus oryzae, Zygorrhynchus moelleri, Z. heterogamus,* and *Umbelopsis isabellina*) and the order *Mortierellales* (*Mortierella* sp.). As much as 10–30% of the total amount of fungal mycelium remains viable for a long time (one month) under anaerobic conditions.

Keywords: facultatively anaerobic fungi, diversity of soil fungi, growth rate.

DOI: 10.1134/S002626170801013X

Soil is a habitat where reductive conditions are often present; in bog and floodplain soils, they may predominate for a long period [1]. Bacteria are traditionally considered the only organisms which remain active in soils under conditions of limited oxygen supply. At the same time, it is known that both yeast and mycelial microscopic fungi have the capacity for fermentation. They can therefore remain active under conditions of limited oxygen supply or in the absence of oxygen and the presence of easy-to-access sugars [2–6].

Fungal biomass is often predominant in soils; therefore, they need to survive at low partial pressure of oxygen or under anaerobic conditions. In most cases, mycelial micromycetes have been considered aerobic organisms. The data on the species diversity of facultative anaerobic fungi, their abundance, and occurrence in various soils are extremely scarce. There have been several reports on the isolation of microscopic fungi (*Fusarium solani* and *Trichoderma harzianum*) from soil, peat, and organic matter samples under anaerobic conditions [7–9].

The aim of the present work was to assess the abundance and diversity of microscopic fungi isolated from various soils under anaerobic conditions.

[1] Corresponding author; e-mail: kurakov57@mail.ru

MATERIALS AND METHODS

The samples of the upper horizons of zonal soils were investigated (soddy podzolic, grey forest, and dark grey forest soils, leached, and ordinary chernozems), as well as the samples of peat soils taken from an ombrotrophic peat bog and a minerotrophic fen, of soddy-alluvial and alluvial-meadow floodland soil, saline and alkali-saline soil, compost, mosses in various degrees of decomposition, the intestinal contents and fresh excrement (coprolites) of the earthworms *Lumbricus terrestris*, *Aporrectodea caliginosa* and the muckworm *Eisenia foetida*.

To estimate the mycelial biomass pool in the soils with long-term reduction regime, we used samples of typical soddy gley, peat podzolic gley, and oligotrophic peat soils taken from an ombrotrophic bog under a sparse pine canopy (Central Forest State Nature Biosphere Reserve, Tver oblast). The peat horizons T1 (10–20 cm), T2 (20–40 cm), Tn (40–60 cm) are characterized by a high level of *Sphagnum* decomposition, low pH (3.5–4.2), low ash content (2.4–6.0%), and an absorption capacity of 80–90 mg-equiv/100 g of soil.

The effect of long-term anaerobiosis on the total and viable fungal mycelium was studied in fresh samples of peat oligotrophic soils from the Central Forest State Nature Biosphere Reserve and samples of soddy podzolic reclaimed soil taken from a potato plot at the Soil

Station of the Moscow State University. The samples were taken from the Ap horizon (0–10 cm) with humus and total nitrogen concentrations of 2.7 and 0.24 %, respectively. The pH of the soil was 6.7; the hydrolytic acidity, 2.2 mg-equiv/100 g of soil; the degree of base saturation, 92%.

Anaerobic incubation was carried out in penicillin flasks. In the case of soddy podzolic soil, the samples (3.0 g) were preliminarily passed through a 2-mm sieve; then sterile water was added so that the soil humidity reached 100% of the maximum water capacity, and the flasks were sealed with rubber stoppers and tin caps. The air in the flasks was replaced with argon.

The mycelial biomass content was determined by fluorescence microscopy in the samples before and after incubation for 5–6 weeks under anaerobic conditions. Calcofluor was used as a dye [10]. To assess the length of the viable mycelium, the water–soil suspension was supplemented with FDA (fluorescein diacetate) (1 ml per 100 ml of the suspension) [11].

We used a modified Hungate technique [12] to determine the numbers of colony-forming units (CFU), as well as to isolate pure fungal cultures under anaerobic conditions.

During the preliminary experiments, in was established that inoculation of soil samples into liquid glucose-containing mineral medium with peptone, vitamins, and microelements, and subsequent incubation under anaerobic condition resulted in the predominance of only one species of microscopic fungi. Thus, this approach hampers both the study of fungal diversity (since it complicates the detection of minor species) and the CFU numbers of facultatively anaerobic fungi in soil. Therefore, in our further experiments, we used only solid agarized media placed in flat glass cell culture flasks sealed with rubber stoppers.

Agarized medium placed in small cell culture flasks was incubated for 4–12 days at 24–26°C; antibiotics were introduced in concentrations (200 mg/l) higher than those usually used for inoculation. The air in the cell culture flasks was replaced with nitrogen. Oxygen was removed from nitrogen by passing through a Hungate column packed with heated copper shavings. The solution of mineral salts was boiled in order to remove dissolved oxygen and cooled in a crystallizer with cold water. The solution was gassed with nitrogen preliminarily passed through the column packed with heated copper shavings. After cooling, the solution was supplemented with organic compounds, vitamins, and microelements. The medium was poured into the glass cell culture flasks with weighed portions of agar (30 g/l) under anaerobic conditions (under N_2 flow). The oxygen content in the flask atmosphere was determined in a Chrom 3700 gas chromatograph equipped with a thermal conductivity detector. A column (2.0 m) packed with a 5Å molecular sieve was used. The column temperature was 50°C. Argon was used as the carrier gas. Oxygen was either not detected in the flask atmosphere, or, in rare cases, it was detected in trace quantities at the maximum sensitivity of the chromatograph. Methylene blue (*Eh* of the half-transition point is +60 mV at pH 7) was used as an indicator of the oxygen content. The indicator was colored blue, while the oxygen partial pressure was higher than 0.05 atm [13].

Ten cell culture flasks were prepared for each type of soil and inoculated under anaerobic conditions; under aerobic conditions, 10–20 petri dishes were inoculated.

The cultures of fungi were isolated from fresh soil samples by inoculating fine-grained soil on a solid nutrient medium under anaerobic conditions. To isolate the fungal cultures, we used a glucose–peptone medium of the following composition (g/l): KH_2PO_4, 1.0; KCl, 0.5; $MgSO_4$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $(NH_4)_2SO_4$, 2.0; agar, 15.0; peptone, 5.0; glucose, 5.0; and yeast extract, 0.5. The medium was supplemented with a microelements solution (1.0 ml/l) containing the following (mg/l): $FeCl_2 \cdot 7H_2O$, 200; $ZnCl_2 \cdot 7H_2O$, 10; $MgCl_2 \cdot 4H_2O$, 3.0; H_3BO_4, 30; $CoCl_2 \cdot 6H_2O$, 2.0; $CuCl_2 \cdot 2H_2O$, 1.0; Na_2MoO_4, 3.0; $NiCl_2 \cdot 6H_2O$, 2.0; and EDTA, 500. In addition, the medium was supplemented with a vitamin solution (1.0 ml/l) containing (mg/l): 4-aminobenzoic acid, 25; D-biotin, 100; nicotinic acid, 25; calcium pantothenate, 25; pyridoxine hydrochloride, 25; folic acid, 10; riboflavin, 25; B_{12}, 0.5; and lipoic acid, 25. To inhibit bacterial growth in soil inocula, antibiotics (streptomycin and chloramphenicol) were added to the molten cooled sterile medium in a concentration of 200 mg/l of medium. The sterile vitamin, antibiotic, and microelement solutions were added after autoclaving. Difco Bacto agar was used; other components were manufactured by Sigma.

Some soils (soddy podzolic, leached chernozem, etc.) were also inoculated on wort agar and Czapek's medium.

To determine the cell numbers of facultatively anaerobic fungi in soil samples, the media were inoculated with the 1 : 100, 1 : 50, and 1 : 10 dilutions of the water–soil suspension. The inoculum (1.0 ml) was spread evenly over the agar surface by gentle rocking of the flask. For the isolation of pure cultures, inoculation of the media with diluted suspensions was less productive than that with fine-grained soil samples. Inoculation with dilutions higher than 1 : 100 or 1 : 50 yielded few colonies, whereas the probability of contamination of the obtained culture with the soil particles attached to the mycelium increased when lesser dilutions (1 : 10) were applied. When using fine-grained soil as inoculum, the colonies spread from the soil particles to the surface of clean medium; their isolation as pure cultures was therefore more reliable. Sometimes, the use of fine-grained soil made it possible to isolate fungi directly from soil, whereas inoculation of the media with dilutions did not.

The isolation of fungi from the ombrotrophic bog (Central Forest State Nature Biosphere Reserve) was

Table 1. Length of fungal mycelium in peat and soddy alpha humic soils

Soil, horizon (cm), ecosystem	Mycelium length, m/g		Viable mycelium, %
	with calcofluor	with FDA	
Typical soddy gley soil, AY(A_d), hydromorphic meadow	600 ± 73	570 ± 60	95
Peat podzolic gley soil, TJ, fir wood	1393 ± 145	670 ± 71	48
Peat oligotrophic soil, T1 (10–20 cm), ombrotrophic peat bog	815 ± 117	641 ± 69	79
Oligotrophic peat soil, T2 (20–40 cm), ombrotrophic peat bog	1506 ± 149	1149 ± 124	76
Oligotrophic peat soil, T bottom (40–60 cm), ombrotrophic peat bog	400 ± 57	71 ± 29	18

carried out in the field as well. Inoculated petri dishes with solid media (wort, starvation agar medium, and Hutchinson medium) were subjected to long-term incubation at a depth of 25–40 cm in the water-saturated layer characterized by the reducing conditions and pH of about 4.0.

Isolation of fungi in pure cultures and their storage were carried out on wort agar at 4°C. All the cultures isolated from cell culture flasks with N_2 as the gas phase were capable of aerobic growth on wort agar.

The number of CFU in the studied soils incubated in air atmosphere was determined using Czapek medium, as well as the above-mentioned glucose-containing mineral medium supplemented with vitamins and microelements.

To evaluate the growth capacity and to assess the radial growth rate of fungi under oligotrophic conditions, each fungal culture was inoculated onto a glucose-containing mineral medium by three stabs. The media were similar to the previously described one, but they did not contain peptone, yeast extract, and vitamins, and their glucose concentration varied from 0.001 to 1%. The radial growth rate was determined at the stage of linear growth using the equation $K_r = (r_2 - r_1)/(t_2 - t_1)$, where K_r is the radial growth rate, r_1 and r_2 are the colony radii (mm), and t_1 and t_2 are the respective measurement times [14].

The mycelial biomass of *F. oxysporum* 11dn1 grown under aerobic and anaerobic conditions was grown in 200-ml vials containing the above-mentioned liquid glucose-containing mineral medium (50 ml) on a shaker (150 rpm). Anaerobic conditions were created by replacing the air in the vials with molecular nitrogen. The flasks were inoculated with equal sectors of a 2–3-day fungal colony (mycelium) grown in a flat-bottomed vial. From three flasks incubated under aerobic and anaerobic conditions, the mycelium was periodically harvested and filtered through paper filters in order to separate it from the medium. The harvested mycelium was washed with distilled water to remove the medium. It was then dried to a constant weight at 75°C for 24 h; the mycelium yield was determined on an analytical balance to within 0.1 mg.

Identification of microscopic fungi was carried out according to their cultural and morphological properties using the relevant identification guides for each taxonomic group [15–19].

A total of 35 out of 180 isolates belonging to different taxonomic groups were identified on the basis of PCR amplification, with subsequent sequencing of the amplicons and analysis of the obtained sequences. The sequences were edited with the BioEdit sequence alignment editor. Identification was performed using the GenBank Data system BLAST alignment software package (http://www.ncbi.nlm.nih.gov/BLAST/).

Microsoft Excel and Statistica 6.0 were used for statistical analysis of the results.

RESULTS AND DISCUSSION

The effect of the reductive conditions in soils on the fungal biomass yield and CFU numbers. The length of fungal mycelium in peat soils (characterized by the long-term reductive anaerobic period [1]), ranged from several hundred to 1.5 thousand meters per gram; the length of viable mycelium (FDA staining), from several tens to one thousand meters per gram (Table 1). In the upper horizons, the proportion of viable mycelium ranged from 50 to 95% of its total amount; in the lowermost horizon it did not exceed 20%.

Other authors [20–22] have reported high amounts of fungal mycelium in peat soils and bogs. According to these authors, in minerotrophic and ombrotrophic bogs the length of fungal mycelium decreases by an order of magnitude downward the soil profile from the surface to a one-meter depth. The second largest mycelium stock (544 m/cm^3) was discovered in the sapropelic horizon of a eutrophic peat bog at a great depth (6.5–7 m). The same amount of mycelium (560 m/cm^3) was detected in the layer 0–10. In the peat soil of ombrotrophic bogs, the length of fungal mycelium was 18–433 m/cm^3.

Table 2. Dynamics of the mycelium length in soddy podzolic and oligotrophic peat soil during aerobic and anaerobic incubation

Soil, horizon	Incubation conditions	Mycelium length, m/g soil			
		0 days	7 days	15 days	35 days
Soddy podzolic soil, A1	Aerobic	185*	211	125	80
	Anaerobic	185	127	98	54
Soil of the ombrotrophic peat bog, T1 (10–20 cm)	Aerobic	641	564	328	156
	Anaerobic	641	413	222	250
Soil of the ombrotrophic peat bog, T2 (20–40 cm)	Aerobic	1149	1212	687	327
	Anaerobic	1149	1047	417	360
Soil of the ombrotrophic peat bog, Tn (40–60 cm)	Aerobic	71	126	61	93
	Anaerobic	71	138	98	115

Note: * Relative error is 20–25%; the total mycelium length was determined in soddy podzolic soil (calcofluor staining); the viable mycelium length, in oligotrophic peat soil (fluorescein diacetate staining).

Peat bog soils are the permanent habitat of yeasts. Their average number in peat soils (10^5 CFU/g) is considerably higher than that in mineral soil horizons and corresponds to the density of yeast cells in fresh plant debris [23, 24]. The high relative abundance of typical sugar-utilizing yeasts in peat bog soils (in contrast to other soil types) is noteworthy. A majority of them are capable of surviving under anaerobic conditions due to alcoholic fermentation.

To elucidate the proportion of fungal biomass which remains in soils under reductive conditions, a number of laboratory incubation experiments with samples taken from mineral and peat horizons were performed under conditions of controlled anaerobiosis.

Both the total and the viable mycelial biomass in peat and soddy podzolic soil samples incubated under anaerobic conditions decreased reliably during the first two weeks of incubation (down to 30–40% of the initial content) (Table 2). During the next three weeks, the mycelium content in soils did not change significantly. In the case of the samples taken from the lowermost (40–60 cm) horizon of oligotrophic peat soil, anaerobic incubation did not result in a decrease in mycelial biomass in the first two weeks; throughout the five-week experiment, the biomass remained stable.

During the first weeks of aerobic incubation of soddy podzolic and peat soils, the length of fungal mycelium had a slight tendency to increase or (in some cases) exhibited reliable growth; then it reliably decreased (Table 2).

Therefore, long-term (for five weeks) cultivation of fresh soil samples under anaerobic conditions did not result in the death of fungal mycelium; on the whole, however, the amount of mycelium decreased.

In the samples of ordinary chernozem (control and with the addition of glucose; humidity 25%) incubated under anaerobic conditions, a threefold decrease in the length of the fungal mycelium was detected after seven days of incubation [25]. During the first week of its incubation in sterile soddy podzolic soil supplemented with glucose and nitrates, a slight increase in the mycelium length of *F. oxysporium* was detected, followed by a decrease [26].

After prolonged (12 months) anaerobic incubation of the samples of soddy podzolic soil, fungal spores rather than hyphae were detected by fluorescence microscopy. The number of fungal CFU in these samples was 2500 CFU/g; that is, it decreased by an order of magnitude. No fungal cultures were isolated from these soils inoculated on various media and incubated under anaerobic conditions. It seems likely that, in the absence of oxygen, hyphal fragments rather than spores form new colonies in most cases.

After 35-day incubation of soil samples under anaerobic condition, the number of fungal CFU in them revealed by aerobic plating decreased 1.5–2-fold. At the same time, the number of CFU revealed in fungal cultures in the case of anaerobic plating did not change in the soils incubated under anaerobic conditions for this period (Table 3).

Thus, the results obtained demonstrate that both hydromorphic and automorphic soils (in this case, soddy podzolic soil) are inhabited by fungi capable of surviving and, probably, functioning under anaerobic conditions.

The number of colonies of microscopic fungi isolated from soils under anaerobic conditions. Enumeration of microscopic fungi isolated on nutrient media under anaerobic conditions from various soils revealed that the number of CFU varied from one and a

Table 3. The numbers of microscopic fungi in soil before and after anaerobic incubation of the media inoculated under aerobic and anaerobic conditions for 35 days

Soils	CFU/g soil			
	Aerobic conditions of isolation		Anaerobic conditions of isolation	
	0 days	35 days	0 days	35 days
Soddy-podzolic soil*	23300 ± 10000	15770 ± 7100	200 ± 110	160 ± 80
Soil from the ombrotrophic peat bog	76000 ± 15000	–**	350 ± 190	–
Peat from the minerotrophic fen	30100 ± 13500	9260 ± 4150	150 ± 120	370 ± 160
Leached chernozem	16300 ± 7000	6070 ± 2750	250 ± 200	400 ± 180
Alluvial alkali-saline soil	16000 ± 10300	–	320 ± 200	–

Notes: * The number of CFU after 12-month incubation of soddy podzolic soil on Chapek's medium and in air atmosphere.
** No data.

half to four hundred per gram of soil. This number is 50–200 times lower than the amount of fungal germs in soil revealed after the incubation of soil samples under air. This means that the number of fungal propagules of mycelial origin able to grow under anaerobic conditions was 0.5 to 2.0% of the total number of CFU. All the cultures isolated from the samples incubated under anaerobic conditions grew aerobically on wort agar; they are therefore facultative anaerobes. We have detected some differences in the numbers of CFU of facultatively anaerobic fungi depending on the soil type. The number of germs of these fungi is lower in leached chernozem than that in chernozem–meadow saline soil characterized by hydromorphic and reducing conditions. Their number is noticeably higher in ombrotrophic peat soil, which is distinguished by a large stock of fungal mycelium as compared to minerotrophic peat [27].

It has been found that fresh or frozen samples are required to isolate fungi under anaerobic conditions (frozen samples were stored at 15–18°C for less than one or two months). When the samples of air-dry soils were used for anaerobic inoculations, the number of fungi decreased markedly or, more often, was not detected at all. To detect facultatively anaerobic fungi in these soil samples, we had to incubate them in a moistened state for one or two weeks before inoculation. In the case of long-term air-dry storage of the studied soils, the amount of viable mycelium decreased. This is additional evidence that, under anaerobic conditions, hyphal fragments rather than spores form new fungal colonies.

Species diversity of facultatively anaerobic microscopic fungi in soils and associated habitats. In the course of this work, we have obtained approximately 200 isolates, of which 180 strains were identified on the basis of their cultural and morphological properties. A large majority of these strains were identified to the species (75%) level; some (20%), to the genus level. However, we failed to identify several cultures represented by sterile forms.

A total of 35 cultures (the representatives of different taxa identified on the basis of their cultural and morphological properties) were identified by PCR amplification. The results of PCR analysis coincided almost completely with the results of the genus (100%) and species (95%) level identification that was previously carried out, according to the cultural and morphological properties of the fungi in question. Moreover, using this technique, a number of strains represented by sterile mycelium were identified to the species level.

Three to seven species of facultatively anaerobic fungi were detected in each type of soil. *Mucor hiemalis, M. circinelloides, Rhizopus oryzae, Fusarium solani, F. oxysporum, Trichoderma atroviride, T. polysporum, T. harzianum, T. aureoviride, T. viride*, and *T. koningii* were the fungi most often found in the studied soils. Their relative abundance, i.e., their proportion among all the strains isolated from soils under anaerobic conditions exceeded 15%. The next in relative abundance (7–15%) is the group which includes *Zygorrhynchus moelleri, Z. heterogamus, Gliocladium roseum, Acremonium* sp., *Actinomucor elegans, M. racemosus*, and representatives of the genus *Mortierella.* The group of fungi least commonly isolated from the samples incubated under anaerobic conditions (less than 7% of the relative abundance) was represented by the species *Paecilomyces lilacimus, Humicola grisea, Gliocladium penicilloides, Clonostachys grammicospora, Aspergillus terreus, Tolypocladium inflatum, Acremonium* sp., and *Verticillium lecanii.* We have detected significant differences in the composition and abundance of the identified facultatively anaerobic fungi in various soils. It has been established that, in the population of facultatively anaerobic fungi from chernozem, alkali-saline, and saline soils, *Fusarium* spp. were predominant; in soddy podzolic, grey forest, alluvial-meadow, and minerotrophic peat soils, mucor fungi rather than fusaria were dominant; and in soddy

Table 4. Taxonomic affiliation of facultatively anaerobic microscopic fungi

Division	Order	Family	Genus/speciesc
Ascomycota	*Hypocreales*	*Nectriaceae*	*Fusarium solani* (Mart.) Sacc. (1881)
			F. oxysporum Schlecht. (1824)
			Fusarium sp.
			Clonostachys grammicospora Schroers & Samuels (2001)
			C. rosea (syn *Gliocladium roseum*) (J.C. Gilman & E.V. Abbott) Schroers (2001)
			Acremonium sp., related to *A. kashiense* (*Rhizostilbella hibisci* (Pat.) Seifert, (1985))
		Hypocreaceae	*Gliocladium penicilloides* Corda (1840)
			Trichoderma aureoviride Rifai (1969)
			T. harzianum Rifai (1969)
			T. polysporum (Link) Rifai (1969)
			T. viride Pers. (1794)
			T. koningii Oudem. (1902)
		Clavicipitaceae	*Lecanicillium lecanii* (Zimm.) Zare et W. Gams, (syn *Verticillium lecanii* (Zimm.) Viegas 1939)
			Tolypocladium inflatum W. Gams (1971)
	Eurotiales	*Trichocomaceae*	*Aspergillus terreus* Thom (1918)
			A. niger van Teighem (1867)
			Paecilomyces lilacinus (Thom) Samson (1974)
	Incertae sedis		*Acremonium strictum* W. Gams (1971)
			Humicola grisea Traaen (1914)
Zygomycota	*Mucorales*	*Mucoraceae*	*Actinomucor elegans* (Eidam) C.R. Benj. & Hesselt. (1957)
			Absidia glauca Hagem (1908)
			Mucor circinelloides van Tiegh. (1875)
			M. hiemalis Wehmer (1903)
			M. racemosus Fresen (1850)
			Rhizopus oryzae Went & Prins. Geerl. (1895)
			Zygorrhynchus moelleri Vuill. (1903)
			Z. heterogamus (Vuill.) Vuill. (1903)
		Umbelopsidaceae	*Umbelopsis isabellina* (*Mortierella isabellina* Oudem. (1902))
	Mortierellales	*Mortierellaceae*	*Mortierella* sp.

alluvial and ombrotrophic peat soils, as well as in *Sphagnum* and green mosses, *Trichoderma* spp. were detected.

A total of 18 fungal strains were cultivated in petri dishes submerged in the water column of the ombrotrophic peat bog to the depth of 25–40 cm. In the majority of dishes (six out of ten) with Hutchinson medium and wort agar, as well as on two plates with starvation medium, we detected members of the genus *Trichoderma*, which were represented by scarce microcolonies of pale sterile mycelium. The fungi *Ulocladium botrytis* and *U. atrum* were detected as well; however, unlike the above-mentioned species, subsequent examination did not confirm their growth under anaerobic conditions.

Aspergillus niger, Absidia glauca, Mortierella sp., *Zygorrhynchus* sp., *Mucor circinelloides, M. hiemalis*, and *Mucor* sp. have been isolated from the samples of fresh coprolites and the intestinal contents of earthworms (*Lumbricus terrestris* and *Aporrectodea caliginosa*) and muckworms (*Eisenia foetida*), which were incubated under anaerobic conditions.

All the obtained mitotic fungi are characterized by their relations to the perfect stages [28]. All of them belonged to *Ascomycota*, order *Hypocreales*, family *Nectriaceae* (6 species), *Hypocreaceae* (6 species), *Clavicipitaceae* (2 species), and order *Eurotiales*, family *Trichocomaceae* (3 species) (Table 4). A large majority of other isolates belongs to *Zygomycota*, order *Mucorales*, families *Mucoraceae* (8 species) and

Table 5. Dependence of the fungal radial growth rate on glucose concentration under aerobic and anaerobic conditions

Species/strain	Conditions	K_r, mm/h			
		Glucose, %			
		1	0.1	0.01	0.001
Gliocladium penicilloides an-ch11	Anaerobic	0.09	0.13	0.13	0.09
	Aerobic	0.21	0.17	0.16	0.15
Fusarim oxysporum an6	Anaerobic	0.35	0.20	0.21	0.20
	Aerobic	0.20	0.20	0.18	0.15
Mucor hiemalis an48	Anaerobic	0.36	0.22	0.20	0.20
	Aerobic	0.37	0.30	0.31	0.30
Trichoderma aureoviride an5	Anaerobic	0.38	0.19	0.21	0.18
	Aerobic	0.35	0.21	0.22	0.20

Variation coefficient, 15%.

Umbelopsidaceae (1 species), and order *Mortierellales*, family *Mortierellaceae* (1 species).

Hence, the capability for anaerobic growth is a characteristic trait of mycelial micromycetes belonging to a taxonomically small group (that is, no more than 0.5–5.0% of the total number of fungal species in the studied soils) that includes about 30 species belonging to three families of the class *Zygomycetes* and four families of *Ascomycetes.* The diversity of microscopic fungi isolated from various soils, the plant rhizosphere, and plant debris under aerobic conditions is more pronounced, since several thousand species have been isolated. The detailed analysis of the soil microbiota of each biocenosis results in the isolation of 150–300 species, among which the representatives of the genera *Penicillium* and *Aspergillus* prevail. At the same time, no more than three to seven species were detected under anaerobic conditions in the soil samples taken from each biocenosis. Our results on the diversity of fungi detected under anaerobic conditions are somewhat limited due to the application of the above-mentioned isolation technique; moreover, at a given time a limited number of species is present in soil as viable mycelium. The great species diversity of fungi grown on agarized media in air atmosphere can be attributed to the fact that the majority of fungal colonies develop from spores, which probably does not occur in the case of inoculation under anaerobic conditions.

The radial growth rate of fungi under anaerobic conditions. The replacement of oxygen in the flask atmosphere with nitrogen resulted in a decrease (by 1.5–4 times) in the radial growth rate on the glucose-containing mineral medium for six out of the eight strains studied (*Gliocladium penicilloides* an-ch11, *Fusarium solani* an-ch12, *Mucor hiemalis* an48, *Trichoderma aureoviride* an5, *Zygorrhynchus moelleri* an4, and *Rhizopus oryzae* an2). However, the radial growth rate of *Humicola grisea* an-ch13 did not change; in the case of *F. oxysporum* an6, it increased by a factor of 1.4 under anaerobic conditions. The high radial growth rate of fungi under anaerobic conditions is associated with the substrate search strategy. A decrease in the glucose content in the medium from 1% to 0.01% resulted in a sharp decrease in the growth rate of *R. oryzae.* Under oligotrophic conditions, the radial growth rate of the representatives of the genera *Trichoderma* and *Mucor* also decreased during anaerobiosis; similar changes in the radial growth rate of these organisms were observed under anaerobic conditions (Table 5). However, the radial growth rate of the representatives of the genera *Fusarium, Gliocladium*, and *Humicola* hardly changed when the glucose content in the medium was decreased to the minimum level (0.001%) under both aerobic and anaerobic conditions. It should be noted that the biomass yield was much lower under anaerobic conditions, especially at low glucose concentrations, as compared to that under aerobic conditions.

The data obtained indicate that the ability of fungi to grow under oligotrophic conditions (typical for soils) both aerobically and under conditions of limited oxygen availability can be attributed to certain adaptation mechanisms.

Evaluation of the mycelium yield in the case of *F. oxysporum* 11dn1 growing in liquid glucose-containing mineral medium supplemented with vitamins and microelements revealed that under anaerobic conditions the biomass yield was 20 times lower than under aerobic conditions. Under aerobic conditions, the biomass yield by days 4, 5, and 7 was 20, 57, and 165 mg, respectively; under anaerobic conditions, 2, 5, and 8 mg. This can be explained by the fact that the energy efficiency of substrate phosphorylation is lower than that of oxidative phosphorylation.

The results of this work indicate that, in order to isolate pure culture of microscopic fungi, one can apply the following modification of the Hungate method: thin (2 mm) layers of agarized medium supplemented with elevated concentrations of antibacterial antibiotics in small (150–200-ml) cell culture flasks filled with pure molecular nitrogen or with a nitrogen/CO_2 mixture as the gas phase are inoculated with 10-fold diluted soil samples or fine-grained soil. The flasks are incubated at 20–26°C for 7–14 days. From the permanently water-covered horizons of ombrotrophic peat bogs, facultatively anaerobic fungi can be isolated by submerging petri dishes with nutrient medium in the water column for a long enough time.

The literature contains various reports indicating that, apart from yeasts, some mycelial fungi are able to grow under anaerobic conditions on glucose-containing mineral media, on media with various plant and meat extracts, or with peptone, yeast extract, vitamins, and microelements [2–4, 7]. Reports of successful isolation of mycelial fungi directly from natural ecosystems under anaerobic conditions are scarce. The only known work describes the growth of *F. solani* (Mart.) Sacc. cells around soil particles on the surface of oligotrophic medium with silica gel incubated under anaerobic conditions (the atmosphere consisted of CO_2 or $CO_2 + H_2$) detected by light microscopy [8]. The fungus *T. harzianum* has been isolated under anaerobic conditions from peat samples taken from the ombrotrophic peat bog [7]. The fungus that had been tentatively identified as *F. solani*, was isolated after a long-term (more than 12 months) incubation in N_2 atmosphere in a liquid mineral medium with tissue dyes as a carbon source, and soil samples and organic substrates as inocula [9].

In the present work, the ability of *Trichoderma aureoviride, T. atroviride, T. viride, T. polysporum, Umbelopsis (Mortierella) isabellina, Zygorrhynchus heterogamus, Gliocladium penicilloides, G. roseum, Paecilomyces lilacimus, Humicola grisea, Tolypocladium inflatum, Acremonium strictum, Clonostachys grammicospora, Absidia glauca, Actinomucor elegans, Verticillium lecanii*, and *Aspergillus terreus*, isolated from soil and peat samples as well as from the intestinal contents and coprolites of earthworms, to grow under anaerobic conditions was shown for the first time.

Representatives of the genera *Fusarium, Trichoderma, Mucor, Rhizopus*, and *Zygorrhynchus* were most often isolated from soil samples under anaerobic conditions; members of the genera *Absidia, Actinomucor, Mortierella, Aspergillus, Gliocladium, Tolypocladium, Acremonium, Paecilomyces*, and *Humicola* were less abundant.

The capacity for fermentation is one of the factors that influence the fungi distribution in the habitats with limited oxygen supply. For instance, the percentage of yeast species (*Candida paludigena, C. drymidis, C. sake, Pichia silvicola*, and *P. capsulate*) capable of fermentative metabolism was considerably higher in peat samples than in other plant and soil substrates [23 24]. It is well known that the fungi *Rh. oryzae* and *M. racemosus* have the capacity for fermentation [29, 30]. At the same time, other species isolated from soils using the technique of anaerobic incubation have a capacity for substrate phosphorylation. Mucor fungi belonging to the genera *Rhizopus, Mucor*, and *Zygorrhynchus* showed the maximum activity of ethanol production, which can be compared to the ethanol production activity of the wild yeast strains *S. cerevisiae* DSB-3781 and *Hanseniaspora* sp. 1R. Members of the genera *Trichoderma* and *Fusarium* showed lower activity of ethanol production; the ethanol yield by the members of the genera *Gliocladium* and *Humicola* was minimal. Among other compounds, aside from carbon dioxide, the production of acetate, acetaldehyde, and a number of 3- and 4-carbonic volatile compounds (alcohols, aldehydes, and esters) was detected [6].

It is well known that microscopic fungi grow under oligotrophic conditions in air atmosphere. During anaerobic incubation of facultatively anaerobic fungi on the agarized glucose-containing mineral media not supplemented with peptone, yeast extract, and vitamins, their growth has been also detected at the minimal glucose level (0.001%). That is, these species are able to survive in soil under reductive and oligotrophic conditions.

ACKNOWLEDGMENTS

We are very grateful to E.A. Bonch-Osmolovskaya and T.G. Sokolova for providing the possibility of anaerobic cultivation and isolation of microscopic fungi from soil samples.

This work was supported by the Russian Foundation for Basic Research, project no. 06-04-48557.

REFERENCES

1. *Kompleksnaya khimicheskaya kharakteristika pochv Nechernozem'ya* (Comprehensive Characterization of the Soils of Non-Chernozem Area) Orlov, D.S., Ed., Moscow: Mosc. Gos. Univ., 1987.
2. Bartnicki-Garsia, S., Nickerson, W.J., Thiamine and Nicotinic Acid: Anaerobic Growth Factors for *Mucor rouxii, J. Bacteriol.*, 1961, vol. 82, pp. 142–148.
3. Tabak, H.H. and Cooke, W.B., Growth and Metabolism of Fungi in an Atmosphere of Nitrogen, *Mycologia*, 1968, vol. 69, pp. 115–140.
4. Curtis, P.J., Anaerobic Growth of Fungi, *Trans. Br. Mycol. Soc.*, 1969, vol. 53, pp. 299–302.
5. Gunner, H.B. and Alexander, M., Anaerobic Growth of *Fusarium oxysporum, J. Bacteriol.*, 1964, no. 87, pp. 1309–1316.
6. Lavrent'ev, R.B. and Kurakov, A.V., Activity of Alcohol Fermentation and Diversity of Facultatively Anaerobic Mycelial Micromycetes in Soils, *Biotekhnologiya–okhrane okruzhayushchei sredy* (Biotechnology for Environmental Protection), Trudy MBTs MGU, Moscow: Sport i Kul'tura, 2004, p. 1, pp. 103–108.

7. Sizova, M.V., Popova, L.V., and Panikov, N.S., Anaerobic Growth in *Trichoderma harzianum* Rifai, in *Sovremennye problemy mikologii, al'gologii i fitopatologii. Sbornik trudov mezhdunarodnoi konferentsii, posvyashchennoi 80-letiyu kafedry mikologii i al'gologii Mosk. Gos. Univ. i 90-letiyu so dnya rozhdeniya* (Modern Issues in Mycology, Algology, and Phytopathology, Proc. Int. Conf. 80th Ann. Mycology and Algology Department, Moscow University, and 90th Ann. of M.V. Gorlenko), Moscow, 1998, pp. 277–278.
8. Wainwright, M., Ali, T.A., and Killham, K., Anaerobic Growth of Fungal Mycelium from Soil Particles onto Nutrient-Free Silica Gel, *Mycol. Res.*, 1994, vol. 98, pp. 761–762.
9. Marchant, R., Nigam, P., and Banat, I.M., An Unusual Facultatively Anaerobic Filamentous Fungus Isolated under Prolonged Enrichment Culture Conditions, *Mycol. Res.*, 1994, pp. 757–760.
10. *Metody pochvennoi mikrobiologii i biokhimii* (Methods of Soil Microbiology and Biochemistry), Zvyagintsev, D.G., Ed., Moscow: Mosk. Gos. Univ., 1991.
11. Tsuji, T., Kawasaki, Y., Sekiya, T., and Tanaka, S., A New Fluorescence Staining Assay for Visualizing Living Microorganisms in Soil, *Appl. Environ. Microbiol.*, 1995, vol. 61, pp. 3415–3421.
12. Hungate, R.E., A Role Tube Method for Cultivation of Strict Anaerobes, *Methods in Microbiology*, Norris, I.R. and Ribbons, D.W., Eds., London: Academic, 1969, pp. 117–132.
13. Pimenova, M.N., Grechushkina, N.N., and Azova, L.G., *Rukovodstvo k prakticheskim zanyatiyam po mikrobiologii* (Practical Guide for Microbiology), Moscow: Mosk. Gos. Univ., 1971.
14. Bilai, V.I., *Metody eksperimental'noi mikologii. Spravochnik* (Handbook on Experimental Mycology), Kiev: Naukova Dumka, 1982.
15. Booth, C., *Fusarium. Laboratory Guide to the Identification of the Major Species*, Kew, Surrey: Commonwealth Mycological Inst., 1977.
16. Domsch, K.H., Gams, W., and Anderson, T., *Compendium of Soil Fungi*, vol. 1, IHW-Verlag, 1993.
17. Rifai, M.A., A Revision of the Genus *Trichoderma, Mycol. Paper*, 1962, vol. 116, pp. 3–56.
18. Samuels, G.J., *Trichoderma*–a Review of Biology and Systematics of the Genus, *Mycol. Res.*, 1996, vol. 100, pp. 923–935.
19. Schipper, M.A.A., A Study on Variability in *Mucor hiemalis* and Related Species, *Studies in Mycology*, 1973, no. 4, p. 40.
20. Dobrovol'skaya, T.G., Polyanskaya, L.M., Golovchenko, A.V., Smagina, M.V., and Zvyagintsev, D.G., Microbial Pool in Peat Soils, *Pochvovedenie*, 1991, no. 7, pp. 69–77.
21. Golovchenko, A.V., Semenova, T.A., Polyakova, A.V., and Inisheva, L.I., The Structure of the Micromycete Complexes of Oligotrophic Peat Deposits in the Southern Taiga Subzone of West Siberia, *Mikrobiologiya*, 2002, vol. 71, no. 5, pp. 667–674 [*Microbiology* (Engl. Transl.), vol. 71, no. 5, pp. 575–581].
22. Polyakova, A.V., Chernov, I.Yu., and Panikov, N.S., Yeast Diversity in Hydromorphic Soils with Reference to a Grass-Sphagnum Wetland in Western Siberia and a Hummocky Tundra Region at Cape Barrow (Alaska), *Mikrobiologiya*, 2001, vol. 70, no. 5, pp. 714–720 [*Microbiology* (Engl. Transl.), vol. 70, no. 5, pp. 617–622].
23. Polyakova, A.V., Yeasts in Peat Bog Soils, *Extended Abstract of Cand. Sci. (Biol.) Dissertation*, Moscow, 2002.
24. Polyanskaya, L.M., Milanovskii, E.Yu., and Zvyagintsev, D.G., Experimental Modeling of Microbial Succession in Samples of Chernozem in Aerobic and Anaerobic Conditions, *Pochvovedenie*, 2004, no. 9, pp. 1109–1113 [*Eur. Soil Sci.* (Engl. Transl.), no. 9, pp. 975–979].
25. Kurakov, A.V., Role of Microscopic Fungi in Nitrogen Transformation in Soils, *Perspektivy razvitiya pochvennoi biologii* (Prospects of Soil Biology), Proc. All-Russian Conf. 100th Ann. of E.N.Mishustin, Moscow:, MaksPress, 2001, pp. 133–162.
26. Golovchenko, A.V., Spatial Distribution and Structure of Microbial Complexes of Bog–Forest Ecosystems, *Extended Abstract of Cand. Sci. (Biol.) Dissertation*, Moscow, 1992.
27. Ainsworth & Bisby's Dictionary of the Fungi, 2001.
28. Foster, D., *Khimicheskaya deyatel'nost' gribov* (Chemical Activity of Fungi), Moscow: Inostrannaya literatura, 1950.
29. Wainwright, M., Metabolic Diversity of Fungi in Relation To Growth and Mineral Cycling in Soil–a Review, *Trans. Brit. Mycol. Soc.*, 1988, vol. 90, pp. 159–170.