ISSN 1064-2293, Eurasian Soil Science, 2019, Vol. 52, No. 3, pp. 319–332. © Pleiades Publishing, Ltd., 2019.
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SOIL BIOLOGY

The Structure of Bacterial and Fungal Communities in the Rhizosphere and Root-Free Loci of Gray Forest Soil

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Received February 14, 2018; revised February 14, 2018; accepted September 26, 2018

Abstract—The taxonomic composition, abundance, and diversity of bacterial and fungal communities in the rhizosphere loci and bulk mass of the gray forest soil (Eutric Retisol (Loamic, Aric, Cutanic, Humic)) under potatoes, maize, and white mustard grown with the application of mineral and organic fertilizers were compared. Among bacteria, *Bacillus megaterium* and *Paenibacillus polymyxa* species predominated in all the experimental variants. The micromycete community was represented by 39 species belonging to 19 genera. Under the impact of organic fertilizers, the abundance of *Trichoderma harzianum*—an antagonist of many phytopathogens—increased. A decrease in the abundance of representatives of *Fusarium* genus was observed both in the rhizosphere and in the bulk soil. Fertilization was the most significant factor determining the structure and diversity of micromycete communities both in the soil and rhizosphere. The application of mineral fertilizers reduced the diversity of micromycetes in soil, whereas the use of organic fertilizers increased it. In general, organic fertilizers proved to be more favorable for the rhizosphere and bulk soil mycobiomes and for the total soil suppressiveness than the mineral fertilizers.

Keywords: bacteria, micromycetes, maize, mustard, potato, organic fertilizers, mineral fertilizers
DOI: 10.1134/S1064229319010137

INTRODUCTION

The rhizosphere is a narrow soil zone directly adjacent to plant roots, which is formed under the effect of root exudates. The rhizosphere is considered as one of the "hot spots" of the soil microbial activity and belongs to the most complex loci in the diversity and interspecies relations [30, 36]. The composition and abundance of microorganisms in the rhizosphere differ from those in the bulk soil. The rhizosphere microbiome includes nitrogen fixators, rhizobacteria (PGPR), mycorrhizaformers, and many other microorganisms having a favorable effect on the growth and development of plants, as well as phytopathogenic bacteria, fungi, and oomycetes that adversely affect the crop [35]. In the rhizosphere, phytopathogens actively interact with the microbial community, and these interactions determines largely the soil suppressiveness—the capacity to suppress or eliminate some types of pathogens. Therefore, when assessing the suppressive activity of soil, one should focus on characteristics describing the properties of the rhizosphere microbiome or its individual key representatives.

The composition and structure of the rhizosphere microbiome depends on the physicochemical properties of soil (carbon and nitrogen content, pH, texture, etc.), seasonal changes in temperature and moisture [46], species, plant genotype and stage of the plant development [1, 6, 39, 46]. Another factor that determines the microbiome structure of both rhizosphere and bulk soil microbial community is the use of mineral and/or organic fertilizers. The influence of traditional and organic farming systems on the soil microbial diversity is considered to be complex, contradictory, and unclear [27]. The high concentrations of the mineral fertilizers applied to the soil suppress its enzymatic activity [9], modify the soil microbial community in accordance with adaptive responses characteristic of homeostasis, stress, resistance, and repression [4]. The long-term application of mineral nitrogen may reduce the intensity of mycorrhiza development in crop roots [33], decrease the formation of spores and mycelium in arbuscular mycorrhizal fungi [24], as well as limit the species richness of fungi and bacteria due to soil acidification when applying physiologically acid salts [41].

The use of organic fertilizers, such as manure or composts promotes the enrichment of soil with microbial biomass contained in the fertilizer itself and developing in soil due to the addition of carbon and nitrogen [18]. The response of bacteria and fungi to the

organic fertilizers can vary significantly, since most bacteria use only simple and readily available organic substances, whereas many fungi can also decompose complex substrates [34]. In organic farming, the composition of the soil microbiome changes in the direction of increasing the share of *r*-strategists [16]. The functional and taxonomic diversity of the microbial community also increases owing to the growth in the number of taxa—antagonists [22, 43], which results in decreasing abundance of plant pathogens [44].

However, the use of only organic fertilizers, especially liquid and non-contaminated manure, can also be a reason for environmental disturbances in soil ecosystems [38]. First, a large number of microorganisms that are not typical for the soil environment are amended with the manure applied. Second, organic fertilizers are enriched with readily decomposable organic compounds, the presence of which can initiate the development of fast-growing microorganisms with the dominance of phytopathogens. Organic fertilizers can also have an inhibitory effect on species of soil microorganisms that are not adapted to such compounds.

Thus, the rhizosphere is a dynamic locus with a specific soil microbial structure. The significant variation in the composition of the rhizosphere microbiome caused by the ecophysiological rhythms of root exudates during the ontogenesis of different plant species complicates the identification of microorganisms typical for the rhizosphere. One can suggest that rhizosphere and non-rhizosphere microbiomes will differ in their responses to the application of fertilizers. In this study, the abundance and diversity of cultivated bacteria and micromycetes were analyzed in the rhizosphere and non-rhizosphere loci of the gray forest soil with organic and mineral fertilizers under potatoes, mustard, and maize at the different stages of ontogenesis.

OBJECTS AND METHODS

Soil. Samples of gray forest soil (Eutric Retisol (Loamic, Aric, Cutanic, Humic)) collected from the plots of a long-term microfield experiment (Institute of Physicochemical and Biological Problems of Soil Sciences, Russian Academy of Sciences, Pushchino, Moscow oblast) were investigated. The gray forest soil is characterized by a balanced microbial growth in the presence of a substrate [31]. The microbial biomass of gray forest soil is dominated by fungi, and the contribution of bacteria is no more than 9–20% [5, 13]. The conditions of the considered microfield experiment are described in detail in [7]. Soil samples were collected from two variants of the experiment, where for seven years a full mineral fertilizer was applied annually at doses of N180P180K180 (urea, superphosphate, potassium sulfate) or fresh cattle manure at a dose of 50 t/ha. The experimental crops were maize (hybrid Moldavskii 215 MV), potato (Zhukovskii), and white mustard (Raduga). The first soil sampling was carried out in the third decade of June (the phase of 2–4 maize leaves, the beginning of budding in potatoes and flowering in mustard); the second stage was in early August (6–8 maize leaves, the beginning of tuber formation in potato and pods in mustard). The soil samples were collected in two loci: in the rhizosphere of vegetating plants and in the space between the rows (for potatoes, at a distance of 10–15 cm from the planted tuber). The control was the soil of long-term black fallow. All soil samples were indexed according to the scheme: plant–fertilizer system–soil locus–sampling period. The following indices were used for the plants studied: P—potato, W—white mustard, M—maize; the indices for fertilizer systems were M for mineral and O for organic ones; the soil loci had the indices of B for the bulk soil and R for the rhizosphere. The period of sampling was 1 for June and 2 for July. The bare fallow had the BF index (Table 1).

In all the variants, soil samples were collected in three replicates. First, a soil monolith was cut in a row spacing of 5 × 5 × 10 cm, from which three mixed samples were taken. Then, a soil monolith was cut out with a vegetating plant. On a table, large clumps of soil were removed, and rhizosphere soil was shaken off the surface of roots using a brush [10]. Soil samples for microbiological analyses were collected following the requirements of the control for microbiological contamination. Before analyzing, the soil samples were stored in sterile bags for a month in a refrigerator at a temperature of + 4°C. Air-dried samples were used for the analysis of chemical soil properties.

Soil chemical properties and microbial biomass. The moisture weight percentage in the fresh soil samples was determined by their drying at 105°C for 24 h. The content of organic carbon (C_{org}) and of total nitrogen (N_{tot}) were measured using an automatic Leco 932 HCNS-analyzer (USA); pH_{H2O} was detected by potentiometry at the soil: water ratio of 1 : 2.5.

Cultivation on nutrient media. The taxonomic composition of bacteria and microscopic fungi cultivated were determined using the method of microbiological inoculation. Suspensions of the soils (1 : 10 dilution) were prepared in sterile plastic bags. The desorption of bacterial cells was carried out by treating soil suspensions with ultrasound (Branson Digital Sonifier S-450, Branson Ultrasonics, USA) for 2 min at the power of 70% of the maximum one. For microscopic fungi, an MSV-3500 vortex (Latvia) was used at a speed of 3500 rpm for 5 min. An aliquot (100 μL) of a soil suspension was placed on the surface of Czapek sterile agar and potato dextrose agar (PDA) media in Petri dishes with a diameter of 90 mm. Six replicates were used in the experiment. Nystatin (70 mg/L) was added to the media for bacterial cultures to suppress the growth of fungi. The bacterial growth in the fungal cultures was inhibited by the addition of streptomycin (100 mg/L). The number of the bacteria colony-forming units (CFU) grown was counted on the 3rd, 7th,

Table 1. The main chemical properties of soils in the studied variants

Index	Crop	Fertilizer system	Locus	C_{org}	N_{tot}	C/N	pH_{water}
				%			
				June			
PMB-1	Potato	Mineral	Bulk soil	1.23	0.131	9.4	4.51
PMR-1			Rhizosphere	1.34	0.134	10.0	4.53
POB-1		Organic	Bulk soil	1.68	0.153	11.0	5.92
POR-1			Rhizosphere	1.80	0.157	11.5	6.11
WMB-1	Mustard	Mineral	Bulk soil	1.23	0.131	9.4	4.33
WMR-1			Rhizosphere	1.36	0.141	9.7	4.82
WOB-1		Organic	Bulk soil	1.59	0.153	10.4	6.27
WOR-1			Rhizosphere	1.76	0.160	11.0	6.37
MMB-1	Maize	Mineral	Bulk soil	1.22	0.135	9.0	4.44
MMR-1			Rhizosphere	1.35	0.142	9.5	4.56
MOB-1		Organic	Bulk soil	1.67	0.158	10.6	5.75
MOR-1			Rhizosphere	1.81	0.166	10.9	6.20
				July			
PMB-2	Potato	Mineral	Bulk soil	1.22	0.122	10.0	4.39
PMR-2			Rhizosphere	1.35	0.131	10.3	4.41
POB-2		Organic	Bulk soil	1.64	0.157	10.4	6.16
POR-2			Rhizosphere	1.97	0.189	10.4	6.39
WMB-2	Mustard	Mineral	Bulk soil	1.22	0.130	9.3	4.75
WMR-2			Rhizosphere	1.28	0.133	9.6	4.76
WOB-2		Organic	Bulk soil	1.64	0.159	10.3	6.16
WOR-2			Rhizosphere	1.89	0.167	11.3	6.19
MMB-2	Maize	Mineral	Bulk soil	1.21	0.124	9.8	4.40
MMR-2			Rhizosphere	1.33	0.131	10.2	4.58
MOB-2		Organic	Bulk soil	1.58	0.154	10.3	6.15
MOR-2			Rhizosphere	1.82	0.170	10.8	6.35
BF	Bare fallow (control)		Bulk soil	1.01	0.110	9.1	6.12

10th and 14th days, and the number of micromycetes was determined on the 7th, 14th and 20th days. Morphotypes were isolated as pure cultures according to their macro- and microcultural characteristics. The total diversity of fungal communities was estimated by the Shannon index.

Identification of bacterial and fungal strains. Bacterial strains were identified analyzing the nucleotide sequences of 16S rRNA gene. Genomic DNA was extracted using a Proba-Express kit (Syntol, Russia) with the addition of 5% Triton X-100 (AppliChem, Germany). The cell suspension in the lysis solution was boiled at 100°C for 15 min. Then, it was homogenized using glass balls (50–200 μm) on a Mini-Bead-Beater homogenizer (USA) at 5000 rpm for 60 s. Polymerase chain reaction (PCR) was carried out using PCR ScreenMix mixtures (Evrogen, Russia) with primers 27f + Un1492r (strains H1, H8, H9, H10, H12) [17] and 27f + 537r (strains H2–H7, H11) [17, 29]. The PCR products were purified and sequenced according to the Sanger method by the Syntol Company (Moscow, Russia) using the 1100r internal primer (strains H1, H8, H9, H10, and H12) and the 537r primer (strains H2–H7, H11) [29]. The obtained nucleotide sequences were edited using the Chromas Lite 2.01 program (http://www.technelysium.com.au). The alignment, comparison and identification of the nucleotide sequences were carried out using the CLUSTALW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and the BLAST algorithm of the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences obtained were deposited in the NCBI GenBank database under the numbers of MG372733, MG372734, MG372735, MG372736, MG372737, MG372738, MG372739, MG372740, MG372741, MG372742, MG372743, and MG372744.

The specific identification of micromycetes was carried out according to the guides for different groups

of fungi [19, 20]. The taxonomic belonging of individual strains was tested analyzing ITS1 and ITS2 rDNA regions. The DNA isolation was performed according to the procedure described by Glushakova et al. [3] for yeasts, but with three similar cycles, as mycelial fungi are often resistant to temperature changes and action of a lysis buffer. Polymerase chain reaction (PCR) was performed using a PCR mixture ScreenMix (Evrogen, Russia) with the ITSf1 and NL4 primers (Evrogen, Russia). The PCR products were purified and sequenced by the Sanger method by Syntol (Moscow, Russia) using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The reaction products were analyzed on an Applied Biosystems 3130l Genetic Analyzer sequencer using the ITS1f primer.

RESULTS AND DISCUSSION

Chemical properties of the soils. In the soil under all the crops grown, where mineral fertilizers were applied, the C_{org} and N_{tot} contents, C : N ratio, and pH values were lower than in the soil with organic fertilization in both periods of sampling; in the bulk soil (non-rhizosphere loci), compared to the rhizosphere loci, they were also lower (Table 1). When applying NPK, the C_{org} content in the non-rhizosphere and rhizosphere soils was 1.2–1.3 and 1.3–1.4%, respectively, and when applying organic fertilizers, it was 1.5–1.7 and 1.8–2.0%. The N_{tot} content varied from 0.11 to 0.19%, and the C : N ratio ranged from 9.0 to 11.5. The gray forest soil under bare fallow had a slightly acid pH (6.1). The application of organic fertilizers did not change the soil pH; only in some variants, it became less acid. The long-term application of physiologically acid mineral fertilizers, on the contrary, significantly acidified the soil. The increase in the pH values is explained by the excretion of OH^- and HCO_3^- ions by plant roots sorbing nitrates [37].

Abundance and taxonomic composition of bacteria. In the soil of the all variants studied, six *Bacillus* and *Paenibacillus* species (*Firmicutes* phylum, class *Bacilli*) were identified. These gram-positive bacteria are typical representatives of the microbiome in soils of the temperate zone; they are often found in gray forest soils [12]. An unidentified bacterial strain designated by "G" was also revealed, whose cultivation and identification were hampered due to its extremely slow growth.

The number of isolated bacteria species in the soil samples varied from 4 to 7. The largest species diversity (7 species) was detected in the potato–manure–rhizosphere variant in the second term of sampling (POR-2), and in the POR-1, WOB-1, MMB-1, and MMR-2 variants (6 species). The lowest number of identified bacterial species was found in the PMB-1, POB-1 and in bare fallow (BF). The lowest number of identified bacterial species was found in the PMB-1, POB-1 and in bare fallow (BF). The number of cultivated species in the rhizosphere was higher than in the bulk soil or at least the same. A similar pattern was noted in other studied [2]. For all variants with mineral (PMB, PMR, WMB, WMR, MMB, MMR) and organic (POB, POR, WOB, WOR, MOR) fertilization, except for the maize–manure–bulk soil (MOB) variant, the number of species increased to the second period of sampling. This might be due to the increase in root secretions and their more diverse composition when increasing the biomass of plants [45].

The *Bacillus megaterium* and *Paenibacillus polymyxa* species were dominant in the abundance and frequency of occurrence on both nutrient media in all test variants. It is known that these types of bacteria are often found in the rhizosphere of plants. These species appear to be endophytes; they are able to colonize the surface of roots [23]. *Bacillus pumilus* can be referred to rare species, whose presence was revealed only in the WOB-1 and POR-2 variants. *Bacillus mycoides* was not detected in the soil under mustard in any of the variants of the rhizosphere or bulk soil, and *B. pumilus* was found under maize.

Some species of bacteria were cultivated exclusively (*B. pumilus* and G) or predominantly (*B. mycoides* and *B. simplex*) on the potato dextrose agar medium (PDA), which indirectly indicates the relationship of these strains to the root exudates of plants. Nevertheless, the isolated species did not have strict locus preferences. As a rule, in the variants with organic fertilizers and in the rhizosphere, a greater number of species were found than when using the mineral fertilizers and in the non-rhizosphere soil. In the soil of the fallow, the same species that dominated in all other experimental variants (*B. megaterium, B. subtilis,* and *Paenibacillus polymyxa)* were identified.

The total number of colony-forming units of bacteria was about 10^5 CFU/g soil in all the variants, which corresponds to the data usually obtained using the cultivation method for gray forest soils [8]. Nevertheless, the selection of special media and cultivation conditions can increase the number of CFUs in gray forest soils to 10^7 CFU/g soil [25, 42]. The maximum abundance of bacteria on the Czapek medium was found in the maize–NPK–bulk soil (MMB-2) and potato–NPK–bulk soil (PMB-1) variants (4.5×10^5 and 4.0×10^5 CFU/g soil, respectively). In these samples, the maximum number was also found on the PDA medium. The lowest abundance of bacteria on the Czapek medium was revealed for the variants with mineral fertilization in the second period of sampling: mustard–NPK–bulk soil (MMB-2), maize–NPK–bulk soil (MMB-2), and potato–NPK–bulk soil (PMB-2), where $1.2–1.6 \times 10^5$ CFU/g soil were found. On the PDA medium, the highest abundance of bacteria (5.8×10^5 CFU/g soil) was revealed in the potato–NPK–bulk soil (PMB-1) variant, and the minimum in the mustard–NPK–bulk soil (MMB-1)

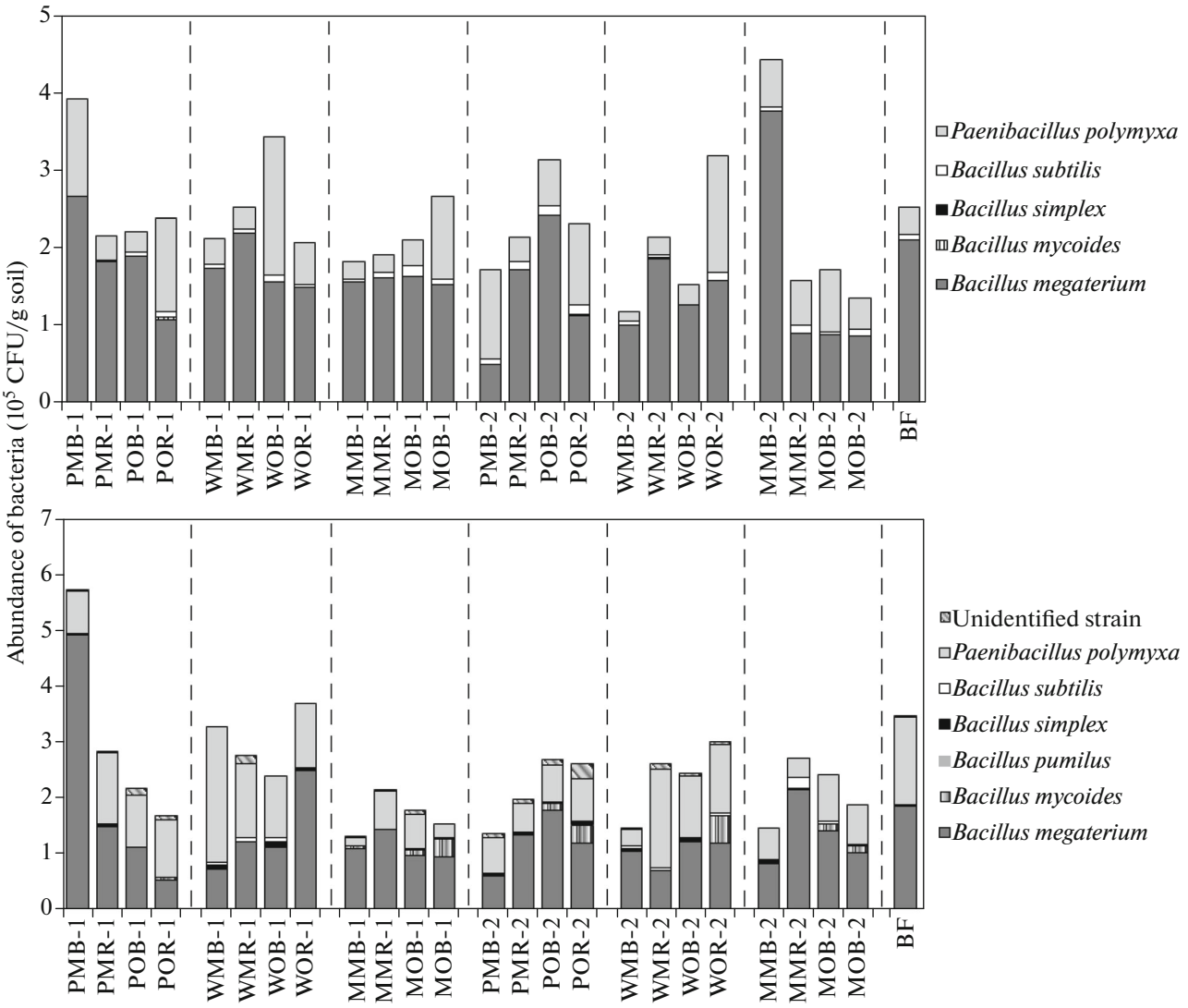

Fig. 1. The taxonomic structure and abundance of the soil bacterial community detected on the Czapek medium (upper figure) and potato dextrose agar (PDA) (lower figure).

and potato–NPK–bulk soil (PMB-2) variants (1.3×10^5 and 1.4×10^5 CFU/g soil, respectively).

Thus, although the low species diversity of the bacterial community was determined on the media used, the results obtained showed the dependence of the growth and development of the *Bacillus* and *Paenibacillus* bacteria on the plant species and phase of its development, as well as on the fertilizer system and soil locus.

Number and taxonomic composition of micromycetes. A total of thirty-nine species of microscopic fungi from nineteen genera of three subdivisions (*Zygomycotina*, *Ascomycotina*, *Deuteromycotina*) were isolated from the soil samples. The subdivision of *Zygomycotina* was represented by species of the *Mucor* and *Rhizopus* genera; the *Ascomycotina*, by species of the *Acremonium*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Clonostachys*, *Epicoccum*, *Fusarium*, *Monilia*, *Mucor*, *Paecilomyces*, *Penicillium*, *Phialophora*, *Phoma*, *Sarocladium, Stachybotrys*, *Talaromyces*, *Trichoderma*, *Verticillium* genera. The most of the isolates were composed of anamorphic fungi—representatives of the *Deuteromycotina* subdivision. The highest diversity was found for *Penicillium* (9 species), *Fusarium* (4 species), *Acremonium* and *Aspergillus* (3 species for each). Only the *Talaromyces* genus was represented by a teleomorph. Five isolates (l/o1, l/o2 (l—light), d/o1, d/o2, d/o 3 (d—dark)) represented by sterile pigmented or hyaline, often yeast-like mycelium were found. Their taxonomic affiliation was not identified from the results of analyzing ITS rDNA due to the formation of low biomass or transition to an uncultivated state in the repeated inoculations.

The number of microscopic fungi species isolated from the soil samples varied from 6 to 25. This great

Table 2. The species composition of soil mycobiome detected on the Czapek (Cz) and PDA (P) media

Species	Variant																								
	PMB-1	PMR-1	POB-1	POR-1	WMB-1	WMR-1	WOB-1	WOR-1	MMB-1	MMR-1	MOB-1	MOR-1	PMB-2	PMR-2	POB-2	POR-2	WMB-2	WMR-2	WOB-2	WOR-2	MMB-2	MMR-2	MOB-2	MOR-2	BF
Acremonium fusidioides[b]				P									P	CzP	P		CzP		CzP			P			
Acr. murorum[b]				P								CzP	P	P	P	CzP	Cz					CzP			
Acr. strictum[a]		CzP							P	Cz	CzP	CzP							P		P	CzP	CzP		
Aspergillus flavus[a,e]						Cz				Cz							Cz								
As. fumigatus[a,e]	Cz			P	P		P	P		P				CzP	P	CzP		P		P	P				
As. versicolor[a,e]											P	Cz							Cz						
As. ustus[a,e]	Cz	Cz	Cz		Cz	Cz	Cz	Cz	CzP	P	CzP	Cz	CzP	Cz	Cz			Cz	Cz	Cz		Cz	Cz		
Aureobasidium pullulans[a,e]				Cz						Cz		P										Cz		Cz	
Cladosporium cladosporioides[a,c,e]												Cz					CzP								
Clad. herbarum[a,c,e]				Cz							P											P			
Clonostachys rosea[a]	Cz			CzP				P	Cz	Cz	CzP			Cz		CzP			Cz		CzP	Cz		CzP	Cz
Epicoccum nigrum[a,c]			Cz				Cz			P	P						CzP					Cz		CzP	
Fusarium dimerum[a,c]		Cz																							
F. oxysporum[a,c]				CzC	P			P		P	P	Cz		Cz			P					CzP		Cz	
F. poae[a,c]		CzP				Cz	CzP		P		CzP	CzP	Cz	CzP	Cz	CzP	CzP	CzP	CzP	C	CzP	CzP	CzP	Cz	Cz
F. solani[a,c]								P				P		Cz								CzP			
Monilia geophila[a]												P				CzP						P		P	
Mucor hiemalis[a,c,e]	Cz	P	P	P	CzP	CzP		P		CzP		P	P		P		P	P			P	P	P		Cz
Paecilomyces lilacinus[a,e]												P													
Paec. variotii[a,e]																								P	
Penicillium canescens[a,c]																	Cz								
P. commune[a]									CzP					P	P	P		Cz			Cz		CzP		Cz
P. corylophilum[a]		P		P					P		Cz		CzP	CzP	P	P	CzP	P	Cz		P	CzP	Cz		CzP

Table 2. (Contd.)

Species	Variant																							
	PMB-1	PMR-1	POB-1	POR-1	WMB-1	WMR-1	WOB-1	WOR-1	MMB-1	MMR-1	MOB-1	MOR-1	PMB-2	PMR-2	POB-2	POR-2	WMB-2	WMR-2	WOB-2	WOR-2	MMB-2	MMR-2	MOB-2	MOR-2	BF
P. funiculosum[a,c]	P	Cz	P	CzP	CzP	P			CzP	P	P	CzP	CzP		CzP		Cz	Cz	CzP	CzP	CzP	Cz	CzP	Cz	CzP
P. griseofulvum[a]	CzP			Cz		CzP	CzP																	Cz	
P. implicatum[a]		P								CzP			Cz												
P. janthinellum[a]	Cz		CzC	P				P		CzP		P	CzP	Cz	CzP		Cz	P	P	CzP		P		P	Cz
P. purpurogenum[a,c]						P	P															Cz			
P. spinulosum[a]	P																								
P. verrucosum[a]	Cz	CzC	P	P	CzC				CzP		P	CzP	CzP	CzP	Cz	Cz	Cz	Cz		Cz	CzP	CzP	CzP	CzP	CzP
P. waksmanii[a]							P								P				P	P					
Phialophora fastigiata[*,a,d]																								CzP	
Phoma exigua[*,a,b,c]																						CzP			
Rhizopus stolonifer[a,d]	P							Cz																	
Sarocladium kiliense[*,b,e]	P			Cz		Cz	P		P		CzP	CzP	Cz		Cz		CzP	Cz	P	Cz	Cz	CzP	Cz		CzP
Stachybotrys chartarum[a,d]		Cz																							
Talaromyces flavus[a]		Cz	CzP	Cz		Cz			Cz		CzP	CzP	P		CzP	CzP		Cz		Cz	Cz		Cz		CzP
Trichoderma harzianum[a,b,d]		CzP	CzP	CzP		P	CzP	CzP	Cz		CzP	CzP	Cz	Cz	Cz	CzP			CzP	CzP	CzP		Cz	CzP	Cz
Verticillium albo-atrum[c,d]															Cz						P	Cz			
Verticillium tenerum[c,d]										CzP															
l/o1								CzP														CzP			
l/o2			Cz																						
d/o1										CzP	CzP											Cz			
d/o2														P		P		P				P			
d/o3																						P			

The taxonomic position was determined by sequencing the ITS rDNA region; a—saprotrophs; b—endophytes, ekkrisotrophs, or inhabitants of rhizosphere; c—potential pathogens of plants; d—cellulolytics; e—conventional pathogens of animals and humans; l/o—light hyaline sterile mycelium; and d/o—dark melanin-pigmented sterile mycelium.

variation is related to the multidirectional influence of ecological and trophic factors in the experimental variants. The highest species diversity was revealed in the maize–NPK–rhizosphere variant in the second sampling period (MMR-2); the minimum diversity was in the mustard–NPK–bulk soil in the first sampling period (WMB-1). The number of fungal species in the rhizosphere was always higher as compared to the bulk soil excluding the variants with potato and mustard when using organic fertilizers in the second sampling period (POB-2, POR-2 and WOB-2, WOR-2).

The number of micromycete species, when using mineral or organic fertilizers, was mostly the same. For all variants with mineral fertilization (PMB, PMR, WMB, WMR, MMB, MMR), the number of microscopic fungi species increased in the second sampling period compared to the first one. This regularity seems to be associated with the phase of plant development and their allelopathic influence on micromycetes. When combining the total number of species in all variants of the experiment, most fungal species were found in the soil under maize (58 species in the sampling period and 62 in the second one). The lowest number of species was found in the soil under mustard (35 species in the first sampling period and 48 species in the second one).

The most common species of microscopic fungi (in 17 out of 25 variants) were *Aspergillus ustus, Fusarium poae, Mucor hiemalis, Penicillium funiculosum, Sarocladium kiliense*, and *Trichoderma harzianum*. Among the rarest species (no more than in three experimental variants) were *Aspergillus flavus, Cladosporium cladosporioides, C. herbarum, Phialophora fastigiata, Verticillium albo-atrum*, and *V. tenerum*, as well as the unidentified l/o1, l/o2, d/o3 isolates. Most of these species are potential phytopathogens [19].

Eight of the 39 isolated fungal species (*Aureobasidium pullulans, Fusarium dimerum, F. solani, Monilia geophila, Verticillium tenerum*, l/o1, d/o2, d/o2) were found only in the rhizosphere of plants. According to other studies, representatives of these species are often ekrisotrophs or phytopathogens [19]. Only three species occurred in the loci without plant roots (bulk soil)—*Penicillium canescens, P. spinulosum,* and c/o2. In addition, the *Fusarium oxysporum* and *Rhizopus stolonifera* species—were found on both media in the rhizosphere; beyond the rhizosphere, they were isolated only on the PDA medium rich in nutrients of plant origin. This fact demonstrates the specificity of trophic conditions in the rhizosphere loci compared to the bulk soil. *Fusarium oxysporum* is a phytopathogen, and *Rhizopus stolonifer* is a cellulolytic. Therefore, their presence in the plant rhizosphere was expected.

The taxonomic composition of micromycetes in the soil under potatoes, mustard, and maize was also different. In the variants with potatoes, the *Aspergillus* genus was represented by only two of four species (all of them were found in the variants with maize and mustard). Instead of the typical for maize and mustard *Fusarium solani, F. dimerum* predominated in the mycobiome of the soil under potatoes. Only in the soil under potatoes, *Stachybotrys chartarum* and *Penicillium spinulosum* species were present, while *P. purpurogenum*, l/o1, and d/o3 were not identified. Many strains of the *Stachybotrys* genus are cellulolytics, and they may exhibit the antagonistic activity against phytopathogenic fungi [19]. In the variants with mustard, unlike the variants under potatoes and maize, several groups of micromycetes were not found: species of the *Aureobasidium*, *Monilia*, and *Verticillium* genera, *Penicillium implicatum* species and sterile morphotypes of mycelium of l/o2, d/o1, and d/o3. Such a wide range of non-present species was apparently caused by the influence of mustard root excretions that had a biocidal effect on some micromycetes [15]. In our study, the *Paecilomyces, Phialophora* and *Phoma* genera, and both identified species of the *Cladosporium* and *Verticillium* genera were micromycetes specific for maize. At the same time, only in the variants under maize, *Rhizopus stolonifer, Penicillium canescens*, and *Penicillium waksmanii* and sterile mycelium of d/o2 isolate were non-present.

The differences in the species composition of the cultivated micromycetes were also typical for different vegetation stages of plants. In the soil under potatoes, in the second sampling period (beginning of tuber formation), *Acremonium strictum, Aureobasidium pullulans, Cladosporium herbarum, Epicoccum nigrum, Fusarium dimerum, Penicillium spinulosum, Rhizopus stolonifer, Stachybotrys chartarum*, and l/o2 were not found. At the same time, *Monilia geophila, Verticillium albo-atrum,* as well as *Penicillium canescens, P. commune, P. waksmanii spedies,* and d/o2 were the new species in the second sampling period. The disappearance of many genera in the rhizosphere and bulk soil in the variants under potatoes may be related to changes in the composition of potato root exudates, as well as to the influence of the fast-growing *Monilia* and *Verticillium* species. In the soil under mustard, in the second period of sampling, *Aspergillus ustus, Fusarium solani, Penicillium griseofulvum, P. purpurogenum, Rhizopus stolonifer* species and l/o1 were not detected, but *Acremonium fusidioides, Ac. murorum, Ac. strictum, Aspergillus versicolor, Cladosporium cladosporioides, Epicoccum nigrum, Penicillium canescens, P. commune, P. corylophilum*, and d/o2 were identified. In the soil under maize, in the second sampling period, *Aspergillus flavus, Cladosporium cladosporioides, Paecilomyces lilacinus, Penicillium implicatum,* and *Verticillium tenerum* were not found, but *Acremonium fusidioides, Paecilomyces variotii, Penicillium griseofulvum, Penicillium purpurogenum, Phialophora fastigiata, Phoma exigua, Verticillium albo-atrum*, and l/o1, d/o1 and d/o2 were determined. In the control soil under the bare fallow, only the species that most often occurred in other experimental variants were detected.

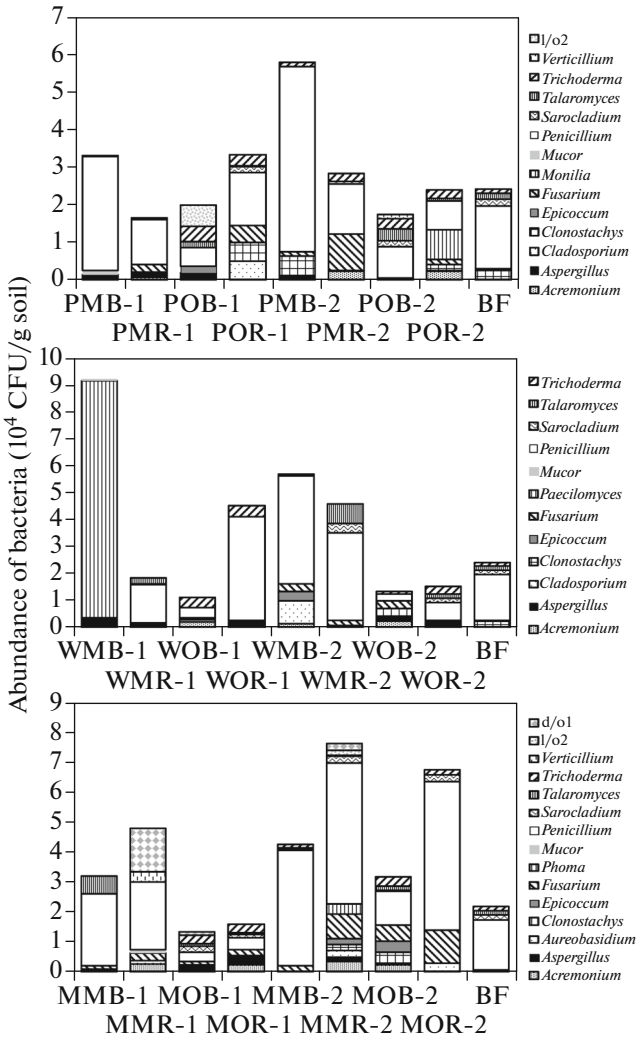

Fig. 2. The taxonomic structure and abundance of the soil mycobiome at the genus rank identified on the PDA medium.

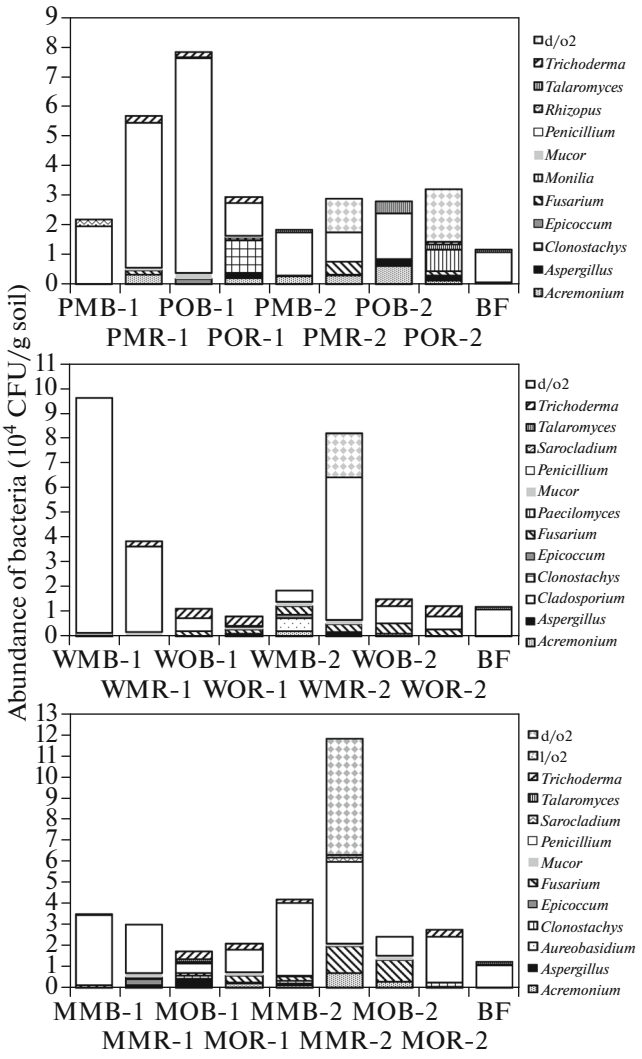

Fig. 3. The taxonomic structure and abundance of the soil mycobiome at the genus rank determined on the Czapek medium in different experimental variants.

In the rhizosphere and bulk soil, when applying organic fertilizers, some specific genera of micromycetes, which were not found in the variants with mineral fertilization, were identified. In the first sampling period, species of the *Acremonium*, *Aureobasidium, Cladosporium,* and *Epicoccum* genera were identified in the soil under potatoes; in the second sampling period, those were *Monilia* and *Verticillium* species. Under mustard, in the variants with organic fertilizers, only the *Clonostachys, Epicoccum,* and *Rhizopus* species were isolated in the first sapling period, as well as *Trichoderma* species werefound in the second one. In the variants with organic fertilization, *Acremonium, Cladosporium, Monilia,* and *Paecilomyces* species were isolated in the first sampling period, while *Paecilomyces* and *Phialophora* species were typical for the second period. Thus, in July, the number of fungal species typical only for the variants with the application of organic fertilizers became significantly less than with mineral fertilization.

The total number of micromycetes in the studied samples was about 10^4 CFU/g soil for all variants (Figs. 2 and 3), which corresponds to the studied on gray forest soils [8]. The largest abundance of micromycetes (9.2×10^4 CFU/g soil) was determined on the Czapek medium in the mustard–NPK–bulk soil (WMB-1) variant. The high number of micromycetes (7.6×10^4 CFU/g soil) on the Czapek medium also characterized the maize–NPK– bulk soil (MMR-2) variant, where the highest abundance of CFUs was found on the PDA medium and also the maize–manure–rhizosphere (MOR-2) variant. The minimum number of microscopic fungi ($1.1–1.6 \times 10^4$ CFU/g) was detected in the mustard–manure–bulk soil (WOB-1, WOB-2) and mustard–manure–rhizosphere (WOR-2) variants on both nutrient media, as

well as in the control (bare fallow) on the PDA medium. The number of CFUs for microscopic fungi in the variants with the use of mineral fertilizers was always higher or equal to that in the variants with organic fertilizers. The number of micromycetes in the rhizosphere of all plants was greater than in the bulk soil, except for the PMB-2, PMR-2 and WMB-2, WMR-2 variants.

The obtained data on the diversity and number of CFUs of microscopic fungi confirm the presence of a hidden negative effect of mineral fertilizers in acid soils [11]. This phenomenon is manifested in a sharp increase in the number of different mycotoxins (ribotoxin, chodicydin, usnic acid, patulin, etc.) in soil due to a sharp increase in the number of *Penicillium funiculosum*, *P. verrucosum* var. *cyclopium*, *P. purpurogenum*, *P. jantinellum*, *Talaromyces flavus*, and *Aspergillus ustus*, replacing the micromycetes typical for these soils. This reorganization of the microbial community leads to a decrease in the yield of agricultural crops without noticeable changes in the agrochemical soil properties [11]. The predominance of these species was revealed in the variants with the application of mineral fertilizers.

The increase in the micromycete species diversity with organic fertilization, as compared to those with applying mineral fertilizers, may be caused by three reasons. First, the application of manure is an effective way to increase the biogenic properties of the soil; it is an important source of active organic matter [7], which leads to the growth and development of copiotrophic microorganisms [16]. Second, the use of mineral fertilizers results in the reorganization of the mycomycete community with the substitution of species common for natural soils. Third, allochthonous microbial species are introduced into the soil with organic fertilizers. It is worth of noting that the problem of the taxonomic structure of the mycobiome in organic fertilizers and its survival, when amended the soil, is still open.

Indicative taxa in soil microbiome. In order to identify indicative taxa, clustering of various experimental variants was carried out using a nonmetric multidimensional scaling (NMDS) and the Bray-Curtis metrics (Fig. 4). A taxon, the number of which on both nutrient media clearly differentiated the pool of the considered experimental variants according to a certain characteristic (plant, soil locus, fertilizer system, term of sampling) was considered as an indicator. As a result of the analysis of these groups, two indicative taxa—*Trichoderma harzianum* and *Fusarium* species—were identified. The *Trichoderma harzianum* species distinctly divided the variants with the application of organic (high abundance of the species) and mineral (low abundance or absence of the species) fertilizers. This species is an antagonist of many phytopathogens [28]. The application of organic fertilizers is known to maintain the growth and high activity of *Trichoderma harzianum* in soil [26]. In our study, the soil of the bare fallows fell into one cluster with the soils, where mineral fertilizers were applied. Therefore, one can conclude that the use of organic fertilizers increases the abundance of *Trichoderma harzianum*, which may positively affect the suppressive activity of the soil against fungal pathogens of plants.

The analysis of the abundance of *Fusarium* species allowed separating the soil under potatoes (where the number of this genus species is higher) and mustard (the number of the genus species is lower) into separate non-crossing clusters, while the variants with maize occupied an intermediate position and crossed with both clusters. Representatives of the *Fusarium* genus are among the most widespread phytopathogenic fungi [19, 32]. Since the soil of the bare fallow fell into one cluster with the soil under potatoes, one can conclude that the presence of mustard reduces the abundance of *Fusarium* species and may increase the suppressive effect of the soil.

Characterization of micromycete alpha-diversity. For soil mycobiomes of the experimental variants, the Shannon index was calculated (Fig. 5) to evaluate the alpha-diversity of fungal communities [14]. The Shannon index ranged from 0.29 and 0.55 for the mustard–NPK–bulk soil (WMB-1) and potato–NPK–bulk soil (PMB-1) variants, respectively, and to 2.94 and 2.75 for the maize–manure–bulk soil (MOB-1) and maize–manure–rhizosphere (MOR-1) variants, respectively. The Shannon index for the mycobiome of the bare fallow was 1.56. The minimum values of the Shannon index were observed in all variants of the bulk soil with the application of mineral fertilizers in both periods of sampling, except for the mustard–NPK–bulk soil (WMB-2) variant. A decrease in alpha-diversity was also found in the mustard rhizosphere, when applying manure (WOR-2). As Fig. 5 shows, the application of mineral fertilizers in five of six variants in the bulk soil, the fungal alpha-diversity strongly decreased (by two–three times). Among all the variants with mineral fertilization, only in the maize rhizosphere (MMR-1 and MMR-2), the alpha-diversity was higher than in the bare fallow. On the contrary, when organic fertilizers were applied in 10 of 12 variants, alpha-diversity indices increased by 1.5–2 times in the bulk soil and rhizosphere loci. The positive effect of organic farming on increasing the diversity and sustainability of the bacterial community was noted in other studies [21].

Unlike the number of colony-forming units in individual taxa or the total number of micromycetes, the assessment of the Shannon diversity index revealed clear trends in the response of the mycobiome, which was mainly associated with the fertilizer system. The use of organic fertilizers resulted in a significant increase of the micromycete diversity in the bulk soil and rhizosphere, since mineral fertilizers had a negative effect on the fungal diversity. The type of

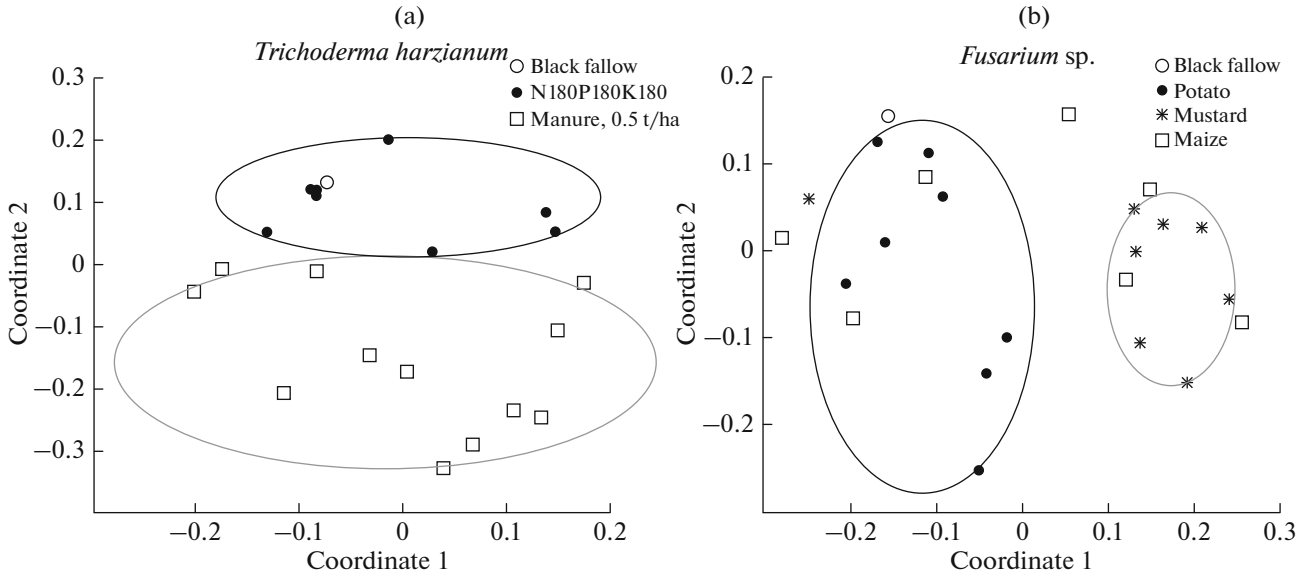

Fig. 4. Clustering of experimental variants based on the abundances of (a) *Trichoderma harzianum* and (b) *Fusarium* sp. on the PDA and Czapek media using the nonmetric multidimensional scaling (NMDS) and Bray–Curtis metrics.

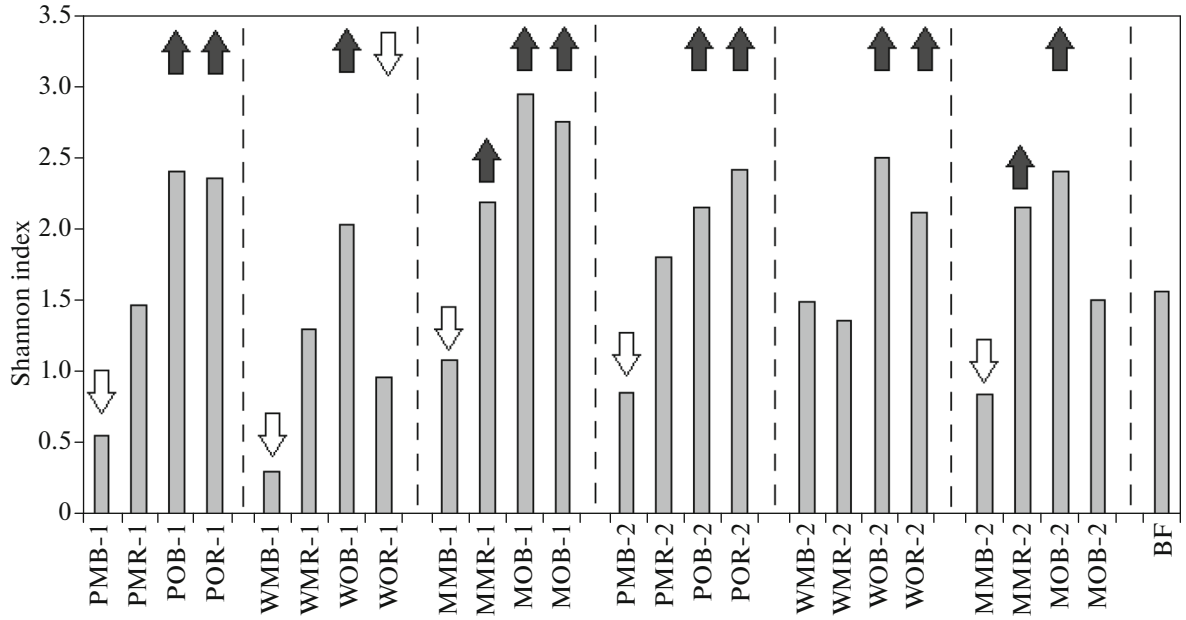

Fig. 5. The alpha-diversity of the soil mycobiomes based on the Shannon index. Gray arrows show the increase and white arrow the decrease in the alpha-diversity relative to the control bare fallow (BF).

soil locus and plant species also played a corrective role in the formation of the taxonomic structure of fungal community. A drastic decrease in the fungal alpha-diversity due to mineral fertilizers was revealed only in the bulk soil, while in the rhizosphere, this effect was mitigated. In the variant with maize, the variety of fungi even increased. Thus, based on the Shannon indices, one can suggest that fertilizer system is the leading factor determining the taxonomic composition and diversity of micromycetes in the bulk soil and rhizosphere.

Characterization of micromycete beta-diversity. The verification of the mycobiomes beta-diversity in different experimental variants was carried out based on the cluster analysis of their similarity using pairwise comparison and the Euclidean metrics (Fig. 6a), as well as nonmetric multidimensional scaling (NMDS) and Bray-Curtis metrics (Fig. 6b). In the first sam-

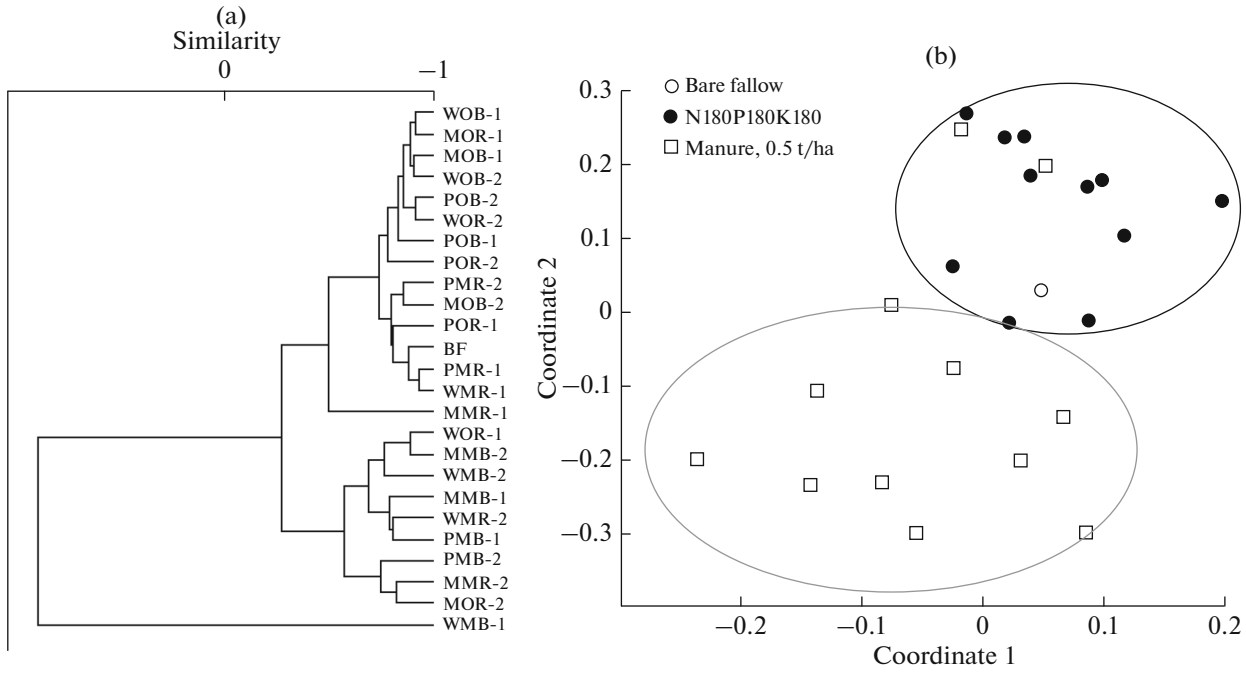

Fig. 6. The β-diversity of the soil mycobiomes: (a) cluster analysis for the similarity of mycobiomes with the use of pairwise comparison and Euclidean metrics and (b) similarity assessment based on the nonmetric multidimensional scaling (NMDS) and Bray–Curtis metrics. The WMB-1 variant significantly differed from the other variants and is not shown in Fig. 6b.

pling period, mustard–NPK–bulk soil (WMB-1) variant differed from the other ones in the mycobiome composition (Fig. 6a). This fact was related to the very high abundance and complete dominance of *Paecilomyces* species that were identified on the Czapek medium only in this variant. For the rest of the experimental variants, mycobiomes of mineral and organic fertilizer systems formed two nun-overlapping clusters (Fig. 6). The upper (Fig. 6a) cluster turned out to be completely formed from the samples with organic fertilization, and the lower one—from those with mineral fertilization, except for two cases with the rhizosphere mycobiomes in the MOR-2 and WOR-1 variants (Fig. 6). The intermediate position, with greater proximity to the variants with organic fertilization, was occupied by soil mycobiomes under bare fallow, as well as rhizosphere mycobiomes under potatoes, mustard, and maize in the soils with NPK (PMR-1, WMR-1, and MMR-1). These results fully agree with the data obtained by the DGGE method on various soils of Japan, which demonstrated a more important role of the fertilization factor forming the soil mycobiome, compared to the soil type [40].

Thus, the data on alpha- and beta-diversity give grounds to consider the fertilizer system as one of the main factors determining the structure and diversity of micromycete communities in the soil and rhizosphere. This means that the application of organic compounds can eliminate the negative processes accompanied by the development of phytotoxicity and serve as an effective method of targeted correction of the composition of the fungal community in the bulk soil and rhizosphere to control the activity of phytopathogens and growth of the overall soil suppressive effects. In addition, the mycobiome abundance and diversity or individual micromycete taxa compared to bacterial communities can be more efficient microbiological indicators of the phytosanitary soil status.

CONCLUSIONS

The analysis of the rhizosphere and non-rhizosphere microbiome in the soils under maize, potato and mustard grown when applying organic and mineral fertilizer systems revealed the indicator taxa of micromycetes— *Trichoderma harzianum* and *Fusarium* sp. The application of organic fertilizers increased the abundance of *Trichoderma harzianum* (an antagonist of many fungal phytopathogens) which could adversely affect the soil suppressive activity. Mustard decreased the abundance of *Fusarium* species both in the rhizosphere and bulk soil.

The fertilizer system was the leading factor determining the composition and diversity of micromycetes in soil, and it was more significant than the location of the soil locus, plant type and the stage of plant development. The application of mineral fertilizers had an adverse effect on the diversity of micromycetes in soil, while organic fertilizers increased fungal diversity in both the bulk soil and the rhizosphere. The application of organic compounds can serve as an effective method to control soil and rhizosphere mycobiomes

and general soil suppressiveness. The micromycete community compared to the bacterial one may be a more precise indicator of the phytosanitary soil status.

ACKNOWLEDGMENTS

This study was supported by the Russian Science Foundation, project no. 17-76-00002.

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Translated by L. Kholopova