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Effect of zinc and copper on cultivable populations of soil fungi with special reference to entomopathogenic fungi

Dalė Pečiulytė*,

Vaidilutė Dirginčiutė-Volodkienė

Institute of Botany of the Nature Research Centre,
Žaliųjų ežerų Str. 49,
LT-08406 Vilnius, Lithuania

The effect of copper and zinc on cultivable soil fungi populations was investigated in a laboratory experiment. Samples of four different soils (arable sandy soil and loam clay; forest sandy soil and forest peat) were collected from sites located in Vilnius district, Lithuania. Metals' effect was elaborated by addition of metal salts ($CuSO_4$ and $ZnSO_4$) at appropriate concentrations into the growth medium (Czapek's agar) and evaluating cultivable fungi abundance and species diversity changes. Zinc or copper salt was added to the medium after its sterilization; zinc concentration varied from 0.05 to 0.20 M (by 0.05 M concentration range) and copper concentration – from 0.5 to 3.0 mM (by 0.15 mM concentration range). At elevated metal salt concentrations, the abundance of cultivable fungi decreased with a marked elimination of some fungi species as compared with a control medium (without metal addition) fungi cultures. Irrespective of a fungi community structure in different type soils, Cu was a stronger inhibitor of soil fungi population abundance than Zn, however, both metals showed a comparable effect on the fungi species diversity. The most resistant fungi belonged to common insect pathogens (*Beuveria bassiana*, *Metarhizium anisopliae*, *Lecanicillium lecanii* and *Isaria* spp.), which dominated comprising up to 90% of all recovered from the soils isolates, due to the metal salt concentration.

Key words: cultivable fungi, entomopathogenic fungi, metal-resistance, zinc, copper

INTRODUCTION

Soil plays an important role as a reservoir of insect-pathogenic fungi, and several species of insect-pathogenic fungi are frequently recorded in cultivated soil worldwide. Fungi from the genera *Beuveria* Vuill., *Metarhizium* Sorokin, *Paecilomyces* Bainier and *Verticillium* Nees are the most common (Keller, Zimmermann, 1989; Klingen, Haukeland, 2006). However, other fungal species, including opportunistic pathogens as well as secondary colonizers, can also greatly affect the dynamics of insect population thriving in soil habitat (Gunde-Cimerman et al., 1998; Ali-Shtayeh et al., 2002; Sun, Liu, 2008). There is evidence that soil pollution with heavy metals can inhibit or kill these fungi. During our earlier investigations of fungal communities in heavy metal-polluted soils a particular heavy metal-resistance of some genera which traditionally are classified as entomopathogenic (Pečiulytė, 2001; Pečiulytė, Dirginčiutė-Volodkienė, 2010; Dirginčiutė-Volodkienė, Pečiulytė, 2011) was noticed. This suggested setting a hypothesis that methods used for the isolation of heavy metal-resistant fungi can be also used for the isolation of insect pathogenic fungi thriving in the soil habitat. The aim of this study was to investigate an impact of increasing zinc and copper concentrations on soil

* Corresponding author. E-mail: dalia.peciulyte@botanika.lt

fungi, cultivable under laboratory conditions with special attention to the populations of the entomopathogenic fungi.

MATERIALS AND METHODS

Soil sampling

The four different soils (arable sandy loam ASL, arable loam-clay ALC, deciduous forest sandy soil – DFSS, and coniferous forest peaty soil – CFPS) selected for the study are located in Vilnius region (at N 54°41'2" longitude and E 25°16'47 latitude, southeastern Lithuania). The mean annual temperature and the average annual precipitation during investigation years are 6.1 °C and 661 mm, respectively. Soil samples were collected in early autumn (12 September, 2008) from 0–10 cm layer after removal of litter. The design involved collection of individual soil samples (per soil type) followed by mixing them into representative samples and analysing those replicates. Each particular soil is characterized by the data measured in four replicates of mixed samples coming from seven individual soil samples from three independent sampling plots. Sampling sites were within 200 m from each other. The composite sample was thoroughly mixed and sample of 500 g was taken for the tests in a laboratory where their pH was measured. The soil samples were placed in PE-bottles, immediately refrigerated at 4 °C, and analysed within one or two weeks. Soil samples were air-dried at room temperature and sieved (2-mm mesh size) prior to further use in the experiment. The arable fields (ASL and ALC) were after the vegetative growth of winter wheat. One forest area was occupied by deciduous forest stands. Dominant tree species were *Fraxinus excelsior* L., *Populus tremula* L., *Betula pendula* Roth., and few stands of *Picea abies* L. H. Karst, *Pinus sylvestris* L. ans *Quercus robur* L. Main bush species: *Juniperus communis* L., *Frangula alnus* Mill., *Sorbus aucuparia* L., and *Padus avium* Mill. The second forest site was occupied by Norway spruce (*Picea abies* [L.] Karst.); mosses and lichens comprised the ground layer, soil contained high organic matter content.

Soil chemical analysis

Analyses were conducted at the laboratory of Institute of Botany of the Nature Research Centre (Vilnius, Lithuania). Soil pH was determined in 1 M KCl extraction – 50 ml of 1 M KCl solution was added to 10 g of soil (six replicates), stirred for 1 hour, left to settle for 30 min and then determined with a pH electrode – WTW 526/538 pH Meter (Germany) (ISO 10390:1994). The concentration of nitrogen and phosphorus was determined with the SPECOL 11 (Carl Zeiss, Jena, Germany) spectrophotometer, potassium with the flame photometer type Flapho 41 after digestion of a dry soil sample with a mixture of HNO_3 and HCl (1:3, v/v). Total concentrations of Zn and Cu in the soils were analysed using extraction of trace elements in aqua regia method (ISO 11466:1994) by electrothermal atomic absorption spectrometry (EAAS) using a Perkin Elmer Zeeman 3030 spectrophotometer. Soil samples for organic matter (OM) content analyses were sieved (2 mm) to remove plant residues and were disaggregated with pestle and mortar. OM content was calculated as the percentage loss of soil weight, after ignition at 550 °C for 4 hours (Howard and Howard, 1990). Soil dry matter was determined by the loss of weight after overnight drying at 105 °C.

All laboratory analyses for geochemical composition of soil were done in four replicates. Soil samples were sieved through a 1-mm sieve. The analytic (<1 mm) part was powdered. Particle size distribution was analysed following a pipette method (Gee and Bauder, 1986). Analysis based on the separation of the mineral soil into various size fractions was only applied to the fine-earth (<2.0 mm) fraction. The procedure involves dispersion, separation of fractions, and determination of sand fractions by dry sieving and determination of silt and clay fractions by pipette analysis (ISRIC FAO, 1995).

Isolation of fungi

Fungi were isolated using a soil dilution method. Isolations were made on Czapek's agar (CA) medium, composed of (g l^{-1}): $NaNO_3$ – 2.0, KH_2PO_4 – 1.0, $MgSO_4 \cdot 7H_2O$ – 0.5, KCl – 0.5, $FeSO_4 \cdot 7H_2O$ – 0.01, glucose – 20.0, agar – 20.0 (pH after sterilization – 6.8) and malt extract agar (2% MEA, Liofilchim, Italy) medium. Chloramphenicol (0.05%) was added to inhibit bacterial growth. The quantification of fungi in soil was carried out by plate counts on different media. Subsamples (10 g) of the soil were diluted 10-fold with sterile saline (0.7% NaCl) and gently agitated

for 10 min. Serial dilutions (1:10) with sterile saline were used to prepare 10^{-2}, 10^{-3} and 10^{-4} soil suspensions. Petri dishes were filled with 100 µL of the appropriate dilution (five replicates each), flooded with 20 ml either of CA and MEA medium of almost 40 °C and incubated at 25 °C. Colonia were counted daily, from day 2 to day 7. Dry weights of samples were used to calculate the actual dilutions.

To test the effects of copper and zinc on the cultivable fungal populations, the media were amended with copper or zinc sulphate. Stock solutions of salts were prepared in distilled and deionised water and filter sterilized. Metal salt was added into the media after their sterilization, pH was adjusted to 6.8 with 1 N sodium hydroxide and 1 N and 10% (v/v) acetic acid. Medium was amended with $ZnSO_4 \times 7H_2O$ and $CuSO_4 \times 5H_2O$ at the following concentrations: 0.025, 0.05, 0.10, 0.15, and 0.20 M for zinc or 0.25, 0.5, 1.0, 2.0, and 3.0 mM for copper sulphates. Plates on the control medium were inoculated with 10^{-3} and 10^{-4} soil suspension dilutions, whereas metal-amended media – with 10^{-1}, 10^{-2} and 10^{-3} dilutions (five replicates each). Colonies were counted after 2–5 days of incubation at 25 °C. The total number of cultivable fungi was expressed as colony forming units (CFU) per gram of dry weight soil. All experiments were conducted in three replicates. The effects of Cu and Zn on soil fungi were evaluated by the tolerance level of the community in the laboratory experiment. The tolerance level (CL_{50}), the heavy metal concentration in growth medium where a fungal CFU decreased to 50% of that in metal unsupplemented medium, was determined for each soil sample and mean semi-lethal concentrations (CL_{50}) for the total investigation were calculated.

Identification of fungi

After isolation and quantification of cultivable fungi, the plates were further incubated in darkness for 10–15 days, and fungi were identified accordingly to general methodological recommendations and fungal classification principles. The inoculums from the emerging fungal colonies grown on the two culture media were transferred to the potato dextrose agar (PDA, Liofilchem, ITALY) slants and maintained at 4 °C until identification. Various selective media: PDA, Sabouroud agar (SA, Liofilchem, ITALY), Czapek Dox agar (CDA, Liofilchem, ITALY), malt extract agar medium (MEA, Liofilchem, ITALY), and Czapek yeast autolysate (CYA, Liofilchem, ITALY) were used for sub-culturing and identification of fungi. All non-sporulating isolates were also grown on water agar (agar 20 g, water 1 000 ml) in an attempt to obtain sporulation. Taxonomic identification was based on morphological characteristics of fungal isolates (shape and size of spores and sporogeneous apparatus) examined with light microscopy. Basic identification keys described in the following books (Ellis, 1971; Watanabe, 1994; Domsch et al., 2007; Kiffer, Morelet, 1999) were used. Genus *Isaria* was identified as assigned following Sung et al. (2007) and Zimmermann (2008). All names of species are cited according to the database of Kirk et al. (2008). The procedure of the fungal isolation and identification was conducted for each soil type, metal salt and concentration.

Statistical analysis

Data of soil chemical properties and cultivable on the appropriate medium fungal populations were systematised and statistically processed through standard analysis of variance (ANOVA). To test for differences between means and LSD-test of significance level was used. The statistical reliability of data was assessed by the absolute limit of least essential difference ($P < 0.05$).

Mean values of the total number of CFU g^{-1} soil and standard deviation was calculated for each soil type, each metal tested and metal salt concentration (n = 15 for each metal-resistance treatment). Percentage of each species in the total number of the fungi isolated either on the control medium, medium amended with zinc or copper sulfate at appropriate concentration was calculated. Shannon-Weiner index (H') was calculated to evaluate the fungus species diversity in the populations grown on the control and zinc- or copper-amended media (Magurran, 1988):

$$H' = -\sum_{i=1}^{S} P_i \hat{u}\ _e P_i, \quad (1)$$

where S is the number of CFU and $P_i = N_i/N$ is the proportion of total samples belonging to the ith CFU. The H' value varies between 0 and $\log_2 S$ and is an information content of relevant sample. The value of H' close to 0 indicates a low diversity, whereas a value close to $\log_2 S$ indicates a high diversity.

Sørensen's index of similarity (S') was plotted to evaluate the impact of different concentrations of $ZnSO_4$ and $CuSO_4$ on the species diversity of

the cultivable soil fungi. The value of *S'* close to 0 indicates a low similarity, whereas a value close to 1 indicates a high similarity (Krebs, 1989):

$$S' = \frac{2c}{a+b}, \quad (2)$$

where *S'* is Sørensen's index of similarity, *a* and *b* are numbers of species in community *a* and *b*, respectively, and *c* is the number of species found in both communities *a* and *b*.

RESULTS

Soil characteristics and chemical properties

The main soil properties are shown in Table 1. In southeastern Lithuania, soils are glacio-fluvial Arenosols, Abeluvisols and Luvisols (ISSS-ISRIC – FAO, 1998). Albeluvisols predominate on soil parent materials of light texture and here distinct erosion processes are prevalent (Buivydaitė and Vaičys, 1996). Prevailing soil texture in arable and deciduous forest horizon is sandy loam, and in subsoil – sand or sandy loam. In places small fields of agriculture land of forests are Podzols (PD) or Dystric Albeluvisols (ABd).

The arable sandy loam (ASL) soil of the present study was characterized by high P content (520.4 ± 0.4 mg kg^{-1} soil), medium K content (143.4 ± 12.8 mg kg^{-1} soil), and low OM content (range: 0.88–0.99%). The granulometric distribution of the ASL by mass was mainly dominated by the sand fraction (range: 51.7–81.3%), with a sandy loam texture. Percentage of silt and clay fractions ranged from 21.3% to 33.5% and from 4.0 to 8.2%, respectively. Total content of nitrogen varied between 0.31% and 0.53%, and soil pH values – from 4.8 to 5.6.

In contrast, the arable loam-clay (ALC) was characterized by low P content (85.75 ± 6.14 mg kg^{-1} soil), high K content (259.34 ± 10.28 mg kg^{-1} soil), and medium OM content (range: 1.5–2.7%). Dominated silt (range: 38.8–79.6%) fraction, followed by clay (range: 35.6–41.0%), and sand ~ 13.0%; soil pH ~ 6.3.

Deciduous forest sandy soil (DFSS) was dominated by the sand fraction (range: 51.7–81.3%), with a sandy loam texture. Percentage of silt ranged from 21.3% to 33.5%, whereas the clay fraction varied from 6.2% to 8.4%. High P content (range: 303.7–357.1 mg kg^{-1} soil), low N content

Table 1. **Selected physical and chemical properties of the soil at investigated sites near Vilnius (Lithuania). Soil samples collected from 0-10 cm layer of horizons A (arable) or Ap (forest).**

Soil management and texture	Soil taxonomy*	Particle size distribution			pH_{KCl}	P (mg kg^{-1} soil)	N (%)	K (mg kg^{-1} soil)	OMC**, %	Zn (mg kg^{-1} soil)	Cu (mg kg^{-1} soil)
		Sand, %	Silt, %	Clay, %							
Arable sandy loam (ASL)	Haplic Luvisols (LVh)	66.5±14.8	27.4±6.1	6.1±2.1	5.2±0.4	520.4±0.4	0.42±0.11	143.4±12.8	0.98±0.1	22.2±0.6	6.5±0.2
Arable loam-clay (ALC)	Dystric Albeluvisols (ABd)	13.0±2.6	59.2±20.4	38.3±2.7	6.3±0.2	85.7 ± 6.2	0.86±0.07	259.3 ± 10.2	2.10±0.6	35.7 ± 1.3	11.4 ± 0.4
Deciduous forest sandy soil (DFSS)	Haplic Arenosols (ARh)	60.4±16.3	32.3±7.4	7.3±1.1	4.5±0.5	330.4±26.7	0.25±0.04	129.6±20.4	1.76±0.5	26.7±0.7	8.2±1.2
Coniferous forest peaty soil (CFPS)	Hapli_Umbric Gleysols (GLu – ha)	ND***	ND	ND	3.8±0.3	32.8±4.2	1.30±0.2	98.7±6.9	45.3±18.7	39.9±1.0	10.6±2.1

*ISSS–ISRIC–FAO (1998) World Reference Basis for Soil Resources. World Soil Resources Report 84. FAO, Rome.

**OMC – organic matter content.

***ND – not determined.

(from 0.21% to 0.29%), medium K content (from 109.2 to 150.0 mg kg^{-1} soil) and acid reaction (pH ~ 4.5) were the main soil characteristic.

Very different soil type, coniferous forest peaty soil (CFPS), rich in organic matter content (range: 26.6–64.0%) was also chosen for the investigation. Soil was characterized by the lowest P and K contents (range: 28.6–37.0 mg kg^{-1} soil and 91.8–105.6 mg kg^{-1} soil, respectively), and the highest N content (range: 1.1–1.5%) as compared with other soil types and by very low soil pH ~ 3.8.

Mean values of zinc (Zn) and copper (Cu) in studied soil samples were 22.2–39.9 and 6.5–11.4 mg kg^{-1} soil, respectively (Table 1). The average concentrations of Zn and Cu were greater in the clayey and peaty soils than in the arable sandy loam and deciduous forest sandy soil. Cu and Zn contents in the ASL were the lowest (~22.2 and 6.5 mg kg^{-1} soil, respectively). In summary, Cu and Zn contents in the soils investigated were comparable to their background concentrations in Lithuanian soils and did not exceed permissible concentrations (Kadūnas et al., 1999; Lithuanian Standard of Hygiene HN60: 2004).

Soil pH in studied soils varied from 3.8 to 6.3 (Table 1). Out of 72 samples analysed, only eighteen (all from CFPS) showed pH values below 3.8. Organic carbon content of the CFPS was ~ 45.3% and it was significantly higher than in other soils studied (Table 1).

Abundance of fungi

Number of fungi grown in control and metal-amended media was calculated as colony forming units (CFUs) per gram of dry weight soil. The percentage of fungi tolerant to each metal at appropriate concentration was also calculated. The highest number of cultivable on control CA medium fungi was isolated from ASL and CFPS samples (~108.29×10^3 and ~113.71×10^3 CFU g^{-1} dw soil, respectively), followed by DFSS (71.35×10^3 CFU g^{-1} soil) and ALC (26.0×10^3 CFU g^{-1} soil) soil samples (Fig. 1).

Numbers of the fungi isolated from different soil types differed significantly ($P < 0.05$) except for ASL and CFPS samples in which the abundance of cultivable fungi was very comparable (108.29×10^3 and 113.71×10^3 CFU g^{-1} soil, respectively). Not only abundance of the cultivable on control medium fungi was different depending on the soil peculiarities. The numbers of Cu- and Zn-resistant fungi isolated from the four types of studied soil also varied due to the soil type and metal concentration. The fractions of metal tolerant fungi in the plates with different $CuSO_4$ and $ZnSO_4$ concentrations are presented in Fig. 2.

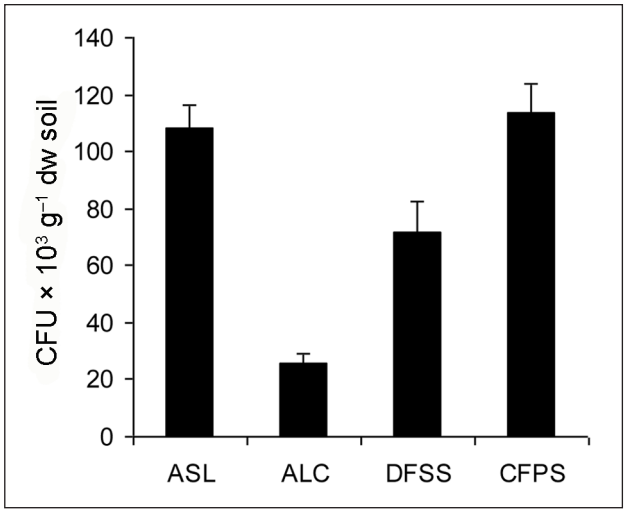


Fig. 1. Abundance (number of CFU) of cultivable on control Czapek's agar medium (without metal additions) fungi. Data presented as mean of five replicates of three separate experiments (n = 15) ± SD (standard deviation). ASL – arable sandy loam, ALC – arable loam-clay, DFSS – deciduous forest sandy soil, CFPS – coniferous forest peaty soil

At low Zn and Cu concentrations in the medium (0.01–0.075vM and 0.1–1.5 mM, respectively), significantly different numbers of the fungal CFUs were determined for both arable (ASL and ALC) and forest soils ($P < 0.05$), while the numbers determined for ASL and CFPS samples did not differ. These two soils distinguished by the comparable abundance of the cultivable fungi on the control medium (Fig. 1) and on the metal-amended media (Fig. 2 A, B). Differences in the abundance of metal-tolerant fungi decreased with the increasing metals (Zn and Cu) concentrations in the isolation medium. Despite these differences, percentage of the metal-tolerant isolates was comparable for all soil types (Fig. 2C, D). A large fraction of cultivable fungi was able to tolerate 0.25 mM Cu and 0.025 M Zn concentrations in the agar media which were several fold higher than the highest content of Cu (11.4 mg kg^{-1}) and Zn (39.9 mg kg^{-1}) found in the soil samples. Stimulating effect of low $ZnSO_4$ concentrations in the nutrient medium on the fungi of

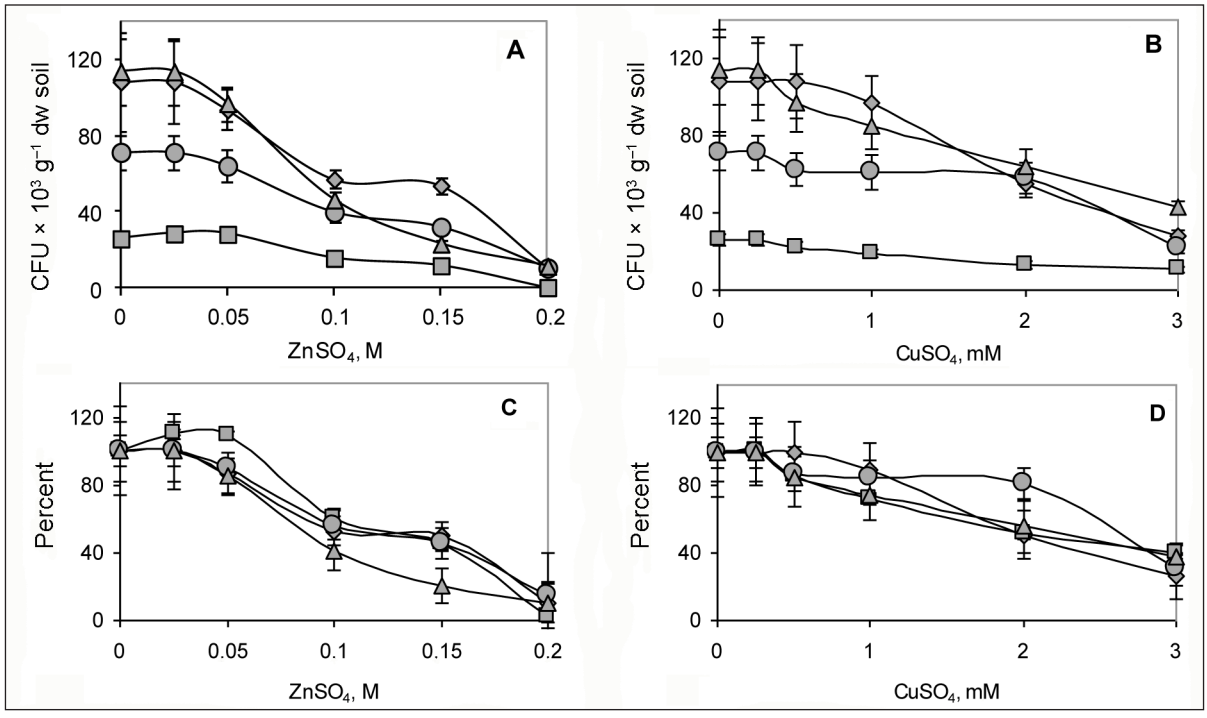


Fig. 2. Abundance (number of CFU g^{-1} dw soil) of metal-resistant fungi cultivable in Czapek's agar medium with appropriate concentrations of $ZnSO_4$ (A, C) and $CuSO_4$ (B, D) and percent of the metal-resistant fungi calculated from their CFU number in control. Data presented as mean of five replicates of three separate experiments (n = 15) ± SD (standard deviation). (–♦–) ASL – arable sandy loam, (–▪–) ALC – arable loam-clay, (–●–) DFSS – deciduous forest sandy soil, and (–▲–) CFPS – coniferous forest peaty soil

ACS samples was noticed. Total fungal tolerance in this soil increased by 10% when Zn concentration was elevated from 0.02 to 0.05 M $ZnSO_4$ (Fig. 2C). Contrariwise, a decrease (by 10%) of the portion of Cu-tolerant fungi was determined when $CuSO_4$ concentration in the medium was elevated from 0.25 to 0.5 mM. The number of Zn-resistant fungi in the medium with 0.1–0.15 M $ZnSO_4$ comprised 53% and 40% for the ASS and ACL soils, respectively (Fig. 2C). Mean semi-lethal $ZnSO_4$ concentration (CL_{50}) for all soil types in the present experiment varied from 0.088 M to 0.15 M (Table 2), and only small portion (9%) of the fungi tolerated 0.2 M $ZnSO_4$ concentration (Fig. 2 C).

Table 2. **The tolerance level – semi-lethal metals (Zn and Cu) concentrations (CL_{50}) for each soil type studied, mean CL_{50} expressed as metal salt and metal ion concentrations, and calculated of each metal concentration equivalent in soil (mg kg^{-1} soil)**

Soil type	Concentration of the metal salt in isolation medium	
	$ZnSO_4 \cdot 7H_2O$	$CuSO_4 \cdot 5H_2O$
Arable sandy soil (ASL)	150 mM	2.01 mM
Arable loam clay (ALC)	139 mM	2.01 mM
Deciduous forest sandy soil (DFSS)	126 mM	2.65 mM
Coniferous forest peaty soil (CFPS)	88 mM	2.37 mM
Mean CL_{50}:		
– of metal salt	(126 ± 20) mM $ZnSO_4 \cdot 7H_2O$	(2.26 ± 0.31) mM $CuSO_4 \cdot 5H_2O$
– of metal ion	(50 ± 10) mM Zn^{2+}	(0.89 ± 0.31) mM Cu^{2+}
[Corresponding to metal ion concentration in soil]	[~3 270 mg Zn^{2+} kg^{-1} soil]	[~57 mg Cu^{2+} kg^{-1} soil]

Traditionally, copper was a stronger fungus-inhibitor than zinc, thus notably lower $CuSO_4$ concentrations were required to reach semi-lethal effects. In the present study, CL_{50} of copper sulphate depended on the soil type and varied from 2.01 mM to 2.65 mM $CuSO_4$ concentration in the isolation medium. The highest CL_{50} (2.65 mM $CuSO_4$) was determined for the fungi from ALC soil. Irrespective of the soil type, the percentage of fungi tolerant to 3 mM $CuSO_4$ concentration remained ≤33%.

Taxonomic distribution

The cultivable on the agar media fungal isolates from four types of soil were assigned to 56 species from Zygomycota (9 species), teleomorphic Ascomycota (1 species), anamorphic (asexual) Ascomycota (45 species), Basidiomycota (1 species), and 3 sterile mycelium forms (Table 3).

The dominant group of cultivable fungi in all soils was mitosporic fungi, represented by Ascomycetes in their anamorphic state and members of Deuteromycota (fungi imperfecti) phylum, which do not have a sexual state or this state has not been discovered. Mitosporic fungi comprised 76.3, 83.3, 72.7 and 61.1% of all strains isolated from ASL, ALC, DFSS and CFPS, respectively (Table 3). Accordingly to their abundance among all isolates, the second group of the fungi was ascribed to the phylum Zygomycota. In different soil types, this group comprised from 13.3 to 18.4% of the total number of isolated fungi. Only one species (namely, *Chaetomium globosum*) belonging to the phylum Ascomycota was isolated from the forest soils (DFSS and CFPS) and one species (*Wallemia sebi*) of the phylum Basidiomycota was isolated from the ASL. The fungal cultivable populations of four different soils contained 3.3–11.1% cultures which did not sporulate (sterile mycelium).

Diversity of fungal populations

*Diversity of fungi cultivable on control medium***.** In total, 56 isolated species from different type soils belonged to 31 genera; the most common were *Penicillium* Link (9 species *Trichoderma* Pers. and *Mucor* P. Micheli ex L. (3 species each), *Cladosporium* Link, *Cunninghamella* Matr., *Isaria* Pers., and *Mortierella* Coem. (2 species each), followed by other 11 genera (1 species each) (Table 4).

The highest diversity of fungal species was determined in ALC (38 species from 25 genera) and DFSS (33 species from 25 genera), followed by ALC (30 species from 22 genera) and CFPS (18 species from 12 genera). It should be noted that species richness and total number of isolated CFU g^{-1} soil were not dependent parameters; correlation coefficients between these two parameters were very low (r_s = 0.20 and – 0.31 for control and Zn-amended isolation medium, respectively), except a negative correlation (r_s = –0.63) determined between the population abundance and species diversity in the Cu-amended media,

Table 3. **Number and percent (%) of fungal genera and species among phyla, evaluated for four soil types studied: arable sandy loam (ASL), arable loam-clay (ALC), deciduous forest sandy soil (DFSS), and coniferous forest peaty soil (CFPS)**

	ASL			ALC			DFSS			CFPS		
	No. of genera	No. of species	%	No. of genera	No. of species	%	No. of genera	No. of species	%	No. of genera	No. of species	%
Zygomycota	4	7	18.4	2	4	13.3	4	6	18.2	2	3	16.7
Ascomycota	0	0	0.0	0	0	0.0	1	1	3.0	1	1	5.6
Basidiomycota	0	0	0.0	0	0	0.0	0	0	0.0	1	1	5.6
Fungi imperfecti (Deuteromycota) (including Ascomycota in their anamorphic state)	19	29	76.3	18	25	83.3	18	24	72.7	6	11	61.1
Mycelia sterilia	2	2	5.3	1	1	3.3	2	2	6.1	1	2	11.1

Table 4. **The list of fungal species isolated from different type of soils on the control medium (without metal salts addition) and the percent of each species in the total number of isolated from the appropriate soil fungi**

Fungus species	Arable sandy loam (ASL)	Arable loam-clay (ALC)	Deciduous forest sandy soil (DFSS)	Coniferous forest peaty soil (CFPS)
Absidia glauca Hagem	2.3 ± 0.2*	6.1 ± 1.3	4.1 ± 1.2	5.3 ± 2.1
Acremonium kiliense Grütz	3.9 ± 0.6			4.4 ± 0.8
Alternaria alternata (Fr.) Keissl.	6.5 ± 2.1	5.4 ± 2.2	0.3 ± 0.1	
Arthrinium phaerospermum Corda M. B. Ellis	1.8 ± 0.4	1.3 ± 0.4		
Aspergillus fumigatus Fresen.	1.1 ± 0.2	4.6 ± 0.8	1.2 ± 0.3	4.9 ± 1.1
Aureobasidium pullulans (de Bary) G. Arnaud				3.4 ± 0.5
Beauveria bassiana (Bals.-Criv.) Vuill.		2.1 ± 0.1		
Botryotrichum piluliferum Sacc. & Marchal		1.9 ± 0.6		
Chaetomium globosum Kunze			1.3 ± 0.6	7.8 ± 2.4
Chromelosporium fulvum (Link) McGinty, Henebert & Kort			0.01 ± 0.0	
Chrysosporium merdarium (Ehrenb.) J. W. Carmich.	1.2 ± 0.2	2.7 ± 0.4	14.3 ± 3.4	
Cladosporium cladosporioides (Fresen.) G. A. de Vries	2.2 ± 0.1	1.8 ± 0.2	0.6 ± 0.1	
Cladosporium herbarum (Pers.) Link	0.4 ± 0.02	0.5 ± 0.03		
Clonostachys rosea f. *rosea* (Link) Schroers, Samuels, Seifert & W. Gams	2.3 ± 0.6	2.7 ± 0.4	3.5 ± 0.2	
Cunninghamella echinulata (Thaxt.) Thaxt. ex Blakeslee	0.02 ± 0.0	3.8 ± 1.6		
Cunninghamella elegans Lendn.			3.4 ± 0.4	
Cylindrocarpon destructans (Zinssm.) Scholten	11.4 ± 1.4	3.5 ± 1.1	2.8 ± 0.5	
Fusarium oxysporum Schltdl.	2.5 ± 0.5		8.6 ± 1.1	
Fusarium solani (Mart.) Sacc.	3.2 ± 0.7			
Gliomastix murorum (Corda) S. Hughes	4.8 ± 0.7			
Humicola grisea Traaen			2.1 ± 0.7	
Isaria farinosa (Holmsk.) Fr. (syn. *Paecilomyces farinosus* (Holmsk.) A. H. S. Br. & G. Sm.)	1.3 ± 0.2	2.6 ± 0.2	0.5 ± 0.1	
Isaria fumosorosea Wize (syn. *Paecilomyces fumosoroseus* (Wize) A. H. S. Br. & G. Sm.)	2.5 ± 0.4	1.2 ± 0.1	0.6 ± 0.1	
Lecanicillium lecani (Zimm.) Zare & W. Gams	0.8 ± 0.1	0.9 ± 0.1	5.3 ± 0.8	
Leptographium sp.		0.6 ± 0.2		
Metarhizium anisopliae (Metschn.) Sorokīn	0.8 ± 0.1			
Mortierella alpina Peyronel	3.2 ± 0.8	5.7 ± 1.2	8.1 ± 1.9	
Mortierella verrucosa Linnem.	2.1 ± 0.4	1.6 ± 0.6	3.2 ± 0.9	
Mucor circinelloides Tiegh.	0.1 ± 0.03		0.2 ± 0.1	0.8 ± 0.3
Mucor hiemalis f. hiemalis Wehmer	0.1 ± 0.02			
Mucor racemosus Fresen.	0.6 ± 0.2		0.7 ± 0.2	2.8 ± 0.7
Paecilomyces carneus (Duché & R. Heim) A. H. S. Br. & G. Sm.			2.2 ± 0.3	
Papulaspora pannosa Hotson			0.09 ± 0.03	
Penicillium chrysogenum Thom	0.7 ± 0.1	2.7 ± 0.2	1.2 ± 0.2	5.5 ± 0.9
Penicillium expansum Link		1.7 ± 0.1		3.9 ± 0.7
Penicillium funiculosum Thom	7.2 ± 1.2	10.2 ± 2.2	8.4 ± 1.3	11.0 ± 1.7
Penicillium glabrum (Wehmer) Westling	1.1 ± 0.2	3.8 ± 0.1		
Penicillium lividum Westling			2.0 ± 0.8	2.6 ± 0.5
Penicillium oxalicum Currie & Thom	0.5 ± 0.1			1.1 ± 0.3
Penicillium restrictum J. C. Gilman & E. V. Abbott	3.5 ± 0.9	8.5 ± 1.7	5.7 ± 1.2	10.5 ± 2.1
Penicillium sp.			1.8 ± 0.4	
Penicillium thomii Maire	6.7 ± 1.8	2.1 ± 0.02		
Phoma exigua Sacc.		4.8 ± 1.0		

Table 4. (Continued)

Scytalidium aurantiacum Klingström & L. Beyer	11.5 ± 3.2	5.1 ± 1.3		
Sporotrichum sp.			0.4 ± 0.0	
Stachybotrys cartarum (Ehrenb) S. Hughes			0.2 ± 0.03	
Tolypocladium sp.		0.7 ± 0.2		
Trichocladium asperum Harz.	0.2 ± 0.03			
Trichoderma harzianum Rifai	3.1 ± 1.1	3.8 ± 1.1	3.8 ± 1.3	1.3 ± 0.4
Trichoderma viride Pers.;	2.1 ± 1.0			
Trichophaea abundans (P. Karst.) Bound.	0.08 ± 0.02			
Ulocladium oudemansii E. G. Simmons			0.1 ± 0.02	
Verticillium albo-atrum Reinke & Berthold	3.0 ± 0.2	1.7 ± 0.2	5.3 ± 0.8	
Verticillium tenerum Nees	0.7 ± 0.1			
Wallemia sebi (Fr.) Arx				8.8 ± 0.3
Zygorhynchus moelleri Vuill.				19.4 ± 0.4
Mycelia sterilia:		2.8 ± 1.2	2.6 ± 0.3	1.4 ± 0.6
Dark (melanized)	1.8 ± 0.4			
White	0.5 ± 0.1		1.8 ± 0.6	5.1 ± 2.2
Number of species	38	30	33	18
Number of genera	25	22	25	12
H′ (Shannon-Wiener index)	3.16	3.18	2.99	2.60

* Percent of fungus species isolates in the total number of isolates (total number of colony forming units, CFU) ± SD (standard deviation).

however, this correlation was statistically insignificant (P = 0.37). The number of species isolated from CFPS was the lowest (18 species), despite of the highest number of the isolated from this soil fungal CFU g^{-1} (113.71 × 10^3 CFU g^{-1}). Three of 18 species, *Aureobasidium pullulants*, *Wallemia sebi* and *Zygorhynchus moelleri*, were isolated only from samples of this soil. Complex of the cultivable on CA medium fungi differed due to the soil type, particularly the complex of the fungi isolated from CFSS sites' soil. This complex can be characterized by the high abundance of some fungal species (8 from 33 species) specific to this soil type. Each of these 8 species belongs to different genera (Table 4).

Consequently, the fungal species richness and community composition depended on selected for the investigation soil type, varying between localities. In the ASL samples dominated *Alternaria alternata*, *Cylindrocarpon destructans*, *Penicillium funiculosum*, *P. thomii* and *Scytalidium aurantiacum*, followed by *Gliomastix murorum*, *Mortierella alpina*, *Fusarium oxysporum*, *F. solani*, *Trichoderma harzianum* and *T. viride* (Table 4). Totally, 36 species isolated from this soil determined relatively high Shannon-Wiener index (H' = 3.16). In the DFSS (H' = 2.99) dominated *Chrysosporium* sp., *Fusarium oxysporum*, *M. alpina*, *P. funiculosum*, *P. restrictum,* followed by *Absidia glauca*, *Cunninghamella elegans*, *T. harzianum* and *Lecanicillium lecani*. Very different and poor in isolated fungal species composition (H' = 2.60) was CFPS; dominated *Chaetomium globosum*, *Penicillium chrysogenum*, *P. funiculosum*, *P. restrictum*, *Zygorhynchus moeleri* and *Wallemia sebi,* followed by *A. glauca*, *Aspergillus fumigatus*, and *Mucor racemosus*. The ALC soil layer could be characterized by the highest richness of fungal species (H' = 3.18). Dominating species in this soil were: *Absidia glauca*, *A. alternata*, *M. alpina*, *P. funiculosum*, *P. restrictum* and *Phoma exiqua*, followed by *Aspergillus fumigatus*, *Cunninghamella echinulata*, *Scytalidium aurantiacum* and *T. harzianum*.

*Zinc- and copper-resistant fungi***.** In total, 18 species belonging to 15 genera were isolated from four types of soil when the medium was amended with 0.126 M zinc sulphate (concentration corresponded mean CL_{50} of zinc sulphate for all soil types) (Table 5).

Som8e fungal isolates (comprising from 1.6 to 13.0% of the number of all analyzed morphotypes) did not sporulate even after they were sub-cultured on the media without Zn additions. The highest number of zinc-resistant species (H' = 2.14) was isolated from ALC (10 species),

Table 5. **Percentage of each fungus species in the total number of fungi isolated from different types of soil samples on Czapek's agar (CA) medium with zinc sulphate (at CL_{50} – 126 mM concentration)**

Fungus species	Arable sandy loam (ASL)	Arable loam-clay (ALC)	Deciduous forest sandy soil (DFSS)	Coniferous forest peaty soil (CFPS)
Aspergillus fumigatus Fresen.				82.2 ± 1.6*
Beauveria bassiana (Bals.-Criv.) Vuill.	1.6 ± 0.2	25.1 ± 1.1		
Clonostachys rosea f. *rosea* (Link) Schroers, Samuels, Seifert & W. Gams	64.5 ± 5.3	6.5 ± 1.2	32.0 ± 2.1	
Cylindrocarpon destructans (Zinssm.) Scholten		6.5 ± 0.6		
Gliomastix murorum (Corda) S. Hughes				
Isaria farinosa (Holmsk.) Fr. (syn. *Paecilomyces farinosus* (Holmsk.) A. H. S. Br. & G. Sm.)	14.0 ± 2.9	4.7 ± 0.7	8.6 ± 0.5	
Isaria fumosorosea Wize (syn. *Paecilomyces fumosoroseus* (Wize) A. H. S. Br. & G. Sm.)	2.2 ± 0.3	2.9 ± 0.2	3.4 ± 0.2	
Lecanicillium lecani (Zimm.) Zare & W. Gams		3.5 ± 0.4		
Leptographium sp. Lagerb. & Melin		2.2 ± 0.6		
Metarhizium anisopliae (Metschn.) Sorokīn			48.2 ± 3.4	
Mortierella alpina Peyronel	3.2 ± 1.1	19.6 ± 3.1		
Mucor hiemalis f. *hiemalis* Wehmer				1.3 ± 0.4
Penicillium expansum Link	8.5 ± 3.1			
Penicillium sp.	4.4 ± 1.2			0.9 ± 0.2
Scytalidium aurantiacum Klingström & L. Beyer		8.7 ± 2.9		
Tolypocladium sp.		7.3 ± 0.5		
Zygorhynchus moeleri Vuill.				15.6 ± 2.0
Others (non-identified: sterile mycelium and other non-sporulating morphotypes**)	1.6 ± 0.9	13.0 ± 0.5	8.2 ± 2.1	
Number of species	7	10	4	4
Number of genera	5	9	3	4
***H'* (Shannon-Wiener index)**	1.23	2.14	1.25	0.55

* Percent of fungus species isolates in the total number of isolates (total number of colony forming units, CFU) ± SD (standard deviation).

** Isolates which did not sporulate after sub-culturing on the media without metal addition.

followed by ASL and DFSS (7 species) (*H'* = 1.23 and 1.25, respectively). The lowest and very poor species diversity of the zinc-resistant fungi was determined in the peaty forest soil (DFSS; 4 species) (*H'* = 0.55). The highest diversity (*H'* = 2.14) (Table 5) in contrast to the lowest total number (11.89×10^3 CFU g^{-1}) (Fig. 1) of the zinc-resistant fungi was determined in the ALC samples with the clear dominance of *B. bassiana* (25.1% of the total number of CFU). Four fungal species from the eleven isolated from this soil type at 0.126 M zinc sulphate concentration in the medium can be identified as opportunistic entomopathogens. In addition to *B. bassiana*, fungi *Isaria farinosa* (syn. *P. farinosus*) (4.7%), *L. lecani* (3.5%), and *Isaria fumosorosea* (syn. *P. fumosoroseus*) (2.9%) were also observed in Zn-resistant fungal populations. Fungi communities of the ASL soils were second in accordance with the diversity of zinc-resistant species (*H'* = 1.23). Three of the seven Zn-resistant species isolated from ASL soil were also ascribed to the group of possible opportunistic entomopathogens: *I. farinosa* (14.0%), *I. fumosorosea* (14.0%) and *B. bassiana* (1.6%). The highest percentage of zinc-resistant entomopathogenic fungi was determined in the DFSS. Four Zn-resistant fungus species dominated in Zn-resistant fungi populations and three of them were entomopathogenic species: high percentage (48.2%) fungus *Metarhizium anisopliae*, followed by *I. farinosa* (8.6%), and *I. fumosorosea* (3.4%). A high percentage of *Clonostachys rosea* f. *rosea* (32.0%) was determined in this soil, too. Complexes of the fungal species isolated from the

CFPS differed from those of other soils: complexes were poor (*H'* = 0.55), composed of four fungal species. *Aspergillus fumigatus* was conspicuous by its abundance (82.2%) and dominance among the Zn-resistant isolates. It is noteworthy that *Zygorhynchus moeleri* also tolerated CL_{50} Zn concentration and was isolated only from CFPS samples. Two species, *Clonostachys rosea* f. *rosea* and *M. alpina*, as well as some non-sporulating morphotypes were distinguished by their occurrence among Zn-resistant fungi (Table 5).

A higher species diversity in the Cu-amended medium (at CL_{50} of $CuSO_4$) than in the Zn-amended medium (at CL_{50} of $ZnSO_4$) was observed in the present investigation. Fungal species able to tolerate copper sulphate at CL_{50} = 2.26 mM concentration are presented in Table 6.

In total, 17 species belonging to 16 genera and a few morphotypes of melanised sterile mycelium were isolated on the Cu-amended medium. The highest diversity of Cu-resistant species (13 species each) was determined in both DFSS (*H'* = 2.19) and ALC (*H'* = 2.28), followed by ASL (10 species) and CFPS (6 species) with *H'* = 1.82 and *H'* = 1.59, respectively. Three well known entomopathogenic species, *B. bassiana*, *M. anisopliae* and *I. fumosorosea,* were the most common among the Cu-resistant fungi, followed by *Paecilomyces lilacinus*. They comprised 68% and 56% of the total number of fungi isolated from DFSS and ASL soil types, respectively. The highest species diversity determined on the control medium for ASL soil (38 species) (Table 4) was highly reduced under copper impact (Table 6). Only 10 of 38 species were able to resist CL_{50} copper sulphate concentration. Two of them, *M. anisopliae*, and *I. fumosorosea,* common entomopathogenic species comprised 5.1% and 15.3% of the total number of isolates, respectively. From thirteen Cu-resistant fungal species isolated from ALC site soil, four species, as in case with Zn-amendments, can be identified as opportunistic entomopathogens; they were *B. bassiana*, *M. anisopliae*,

Table 6. **Percentage of each fungus species in the total number of fungi isolated from different soil type samples on Czapek's agar (CA) medium with copper sulphate (at CL_{50} – 2.26 mM concentration)**

Fungus species	Arable sandy loam (ASL)	Arable loam-clay (ALC)	Deciduous forest sandy soil (DFSS)	Coniferous forest peaty soil (CFPS)
Arthrinium phaerospermum Corda M. B. Ellis	2.0 ± 0.6	1.6 ± 0.4		
Beauveria bassiana (Bals.-Criv.) Vuill.		8.2 ± 0.3	7.6 ± 0.3	
Chaetomium globosum Kunze			1.9 ± 0.6	4.0 ± 1.1
Cladosporium cladosporioides (Fresen.) G. A. de Vries		5.8 ± 0.3	3.8 ± 0.2	
Clonostachys rosea f. *rosea* (Link) Schroers, Samuels, Seifert & W. Gams	2.0 ± 0.3	4.9 ± 0.8	5.4 ± 1.0	
Cunninghamella elegans Lendn.	2.0 ± 0.5	0.8 ± 0.5	1.9 ± 0.6	
Cylindrocarpon destructans (Zinssm.) Scholten		7.4 ± 0.6	3.8 ± 0.3	
Fusarium oxysporum Schtltdl.			1.9 ± 0.5	
Humicola grisea Traaen			1.9 ± 0.5	
Isaria fumosorosea Wize (syn. *Paecilomyces fumosoroseus* (Wize) A. H. S. Br. & G. Sm.)	15.3 ± 2.9	21.3 ± 3.0	22.6 ± 3.5	32.7 ± 4.6
Metarhizium anisopliae (Metschn.) Sorokīn	5.1 ± 0.1	9.8 ± 0.2	17.0 ± 0.4	
Mortierella alpina Peyronel	3.1 ± 0.4			
Paecilomyces lilacinus Thom		16.4 ± 2.4	20.8 ± 2.9	28.6 ± 3.1
Penicillium sp. Link	17.5 ± 4.1	3.3 ± 0.5	3.8 ± 0.7	8.2 ± 1.0
Phoma exiqua Sacc.	21.4 ± 8.2	1.6 ± 7.8		
Scytalidium aurantiacum Klingström & L. Beyer		13.1 ± 3.0		16.3 ± 3.5
Talaromyces luteus (Zukal) C. R. Benj.	1.0 ± 0.2			
Mycelia sterilia (dark)	30.6 ± 9.5	5.8 ± 1.1	7.6 ± 2.1	10.2 ± 3.2
Number of species	10	13	13	6
Number of genera	10	12	12	5
Dark (melanized) fungi (%)	52.04	26.23	13.21	26.53
H' (Shannon-Wiener index)	1.83	2.28	2.19	1.59

I. farinosa and *P. lilacinus* and constituted the great part (in total, 55.7%) of all Cu-resistant fungi isolated from this soil type. Similar dominance of these four species of entomopathogenic fungi was also determined for DFSS. In addition to clear dominance of the Cu-resistant entomopathogenic fungi, a high percentage of fungi capable of synthesizing melanin under Cu impact was also observed. Melanin containing isolates belonging to the species *Arthrinium phaerospermum*, *Cladosporium cladosporioides*, *Phoma exiqua*, *Scytalidium aurantiacum*, and melanised sterile mycelium were noticeable among Cu-resistant soil fungi isolated during the present study. In total, these fungi comprised 13–52% of all fungal CFUs, counted on the nutrient medium amended with 2mM $CuSO_4$ (Table 6). *A. phaerospermum* and *P. exiqua* were more commonly isolated from ASL and ALC soils, *C. cladosporioides* – from ALC and DFSS, *S. aurantiacum* – from ALC and DFPS, while dark (melanised) sterile mycelium forms were frequent in Cu-resistant fungal populations of soil of all types.

Similarity of fungi populations

Different impact of zinc and copper due to their concentration levels on the fungal communities was determined. To emphasize these affects, undamaged fungal populations (control medium cultures) were compared with those impacted by different $ZnSO_4$ and $CuSO_4$ concentrations; Sørensen's similarity indices (*S'*) were calculated and are presented in Table 7.

As disclosed by Sørensen's similarity indices, increased $ZnSO_4$ concentrations in the isolation medium from 0.025 to 0.20 M reduced the diversity of cultivable fungi differently: Sørensen's indices ranged from 1 in all investigated soil types to 0.10 in ASL, to 0.11 and to 0.18 in DFSS and CFPS, respectively, and to 0.38 in ALC. The sharp decrease of the diversity of fungal species isolated from the ASL and DFSS was observed at the $ZnSO_4$ concentrations varying between 0.05 and 0.10 M, in CFPS – between 0.10 and 0.15 M, whereas numbers of species isolated from ALC decreased gradually with the increase of Zn concentrations (Table 7).

Binary similarity between the $CuSO_4$ affected (at concentrations 0.25–3 mM) and the control fungal populations ranged from 1 for all investigated soil types to 0.19 for ASL and CFPS, and to 0.30 and 0.23 for DFSS and ALC, respectively. The sharp decrease of fungus diversity was determined at $CuSO_4$ concentrations varying between 1 and 2 mM for all soils investigated.

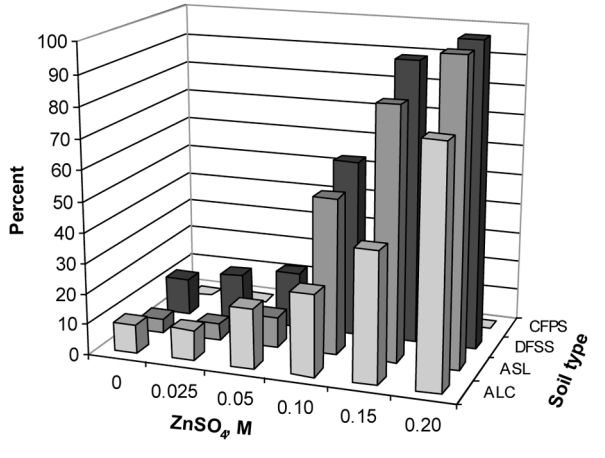
Total percentage of metal-resistant entomopathogenic fungi

In the present investigation, fungal isolates belonging to the species of *B. bassiana*, *M. anisopliae*, *L. lecani*, *I. farinosa* and *C. rosea* were considered as entomopathogenic. Because of a marked dominance of the fungi assigned to that group among zinc and copper resistant fungi, total percentage in these fungi among Cu and Zn-resistant isolates

Table 7. **Similarity coefficients (Sørensen's similarity indices, *S'*) between fungus species complexes in the control medium and medium containing different metal concentrations, depending on the soil type**

Metal sulphate concentration in isolation medium	Arable sandy loam (ASL)	Arable loam-clay (ALC)	Deciduous forest sandy soil (DFSS)	Coniferous forest peaty soil (CFPS)
		$ZnSO_4$		
25 mM	1.00	1.00	1.00	1.00
50 mM	0.88	0.95	0.92	0.88
100 mM	0.29	0.72	0.20	0.62
150 mM	0.27	0.45	0.16	0.18
200 mM	0.10	0.38	0.11	0.18
		$CuSO_4$		
0.25 mM	1.00	1.00	1.00	1.00
0.50 mM	0.97	0.90	0.97	0.94
1.00 mM	0.44	0.56	0.85	0.81
2.00 mM	0.25	0.33	0.43	0.25
3.00 mM	0.19	0.23	0.30	0.19

at appropriate metal concentration was calculated and compared with that of control. The highest abundance of cultivable on control medium entomopathogenic fungi was determined in DFSS (12.1% of the total number of CFU). Their proportion in cultivable fungus populations increased with increasing of $ZnSO_4$ concentration in the medium and reached 100% of all isolates at 0.2 M zinc sulphate concentration (Fig. 3).



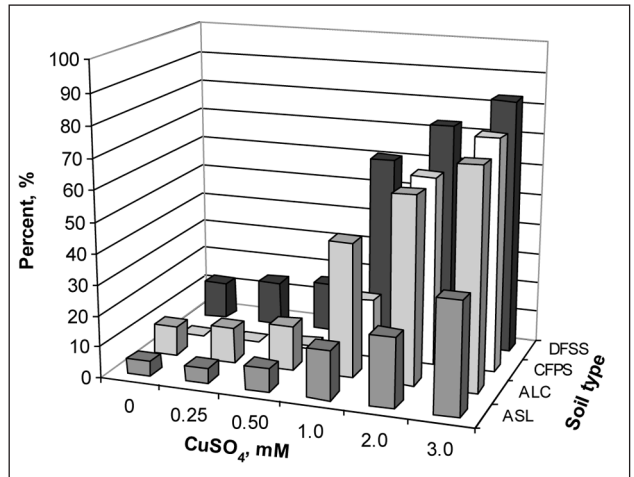
ASL – arable sandy loam, **ALC** – arable loam-clay, **DFSS** – deciduous forest sandy soil, **CFPS** – coniferous forest peaty soil

Fig. 3. Percentage of the CFUs of entomopathogenic fungi in the total number of Zn-tolerant isolates at increasing zinc sulphate concentrations in Czapek's agar medium.

Fraction of the cultivable on the control medium entomopathogens comprised 9.5% of all isolated from ALC soil fungi and that percent increased up to 77.4% under the impact of the highest (0.2 M) $ZnSO_4$ concentration. The poorer in the abundance of entomopathogenic fungi was ASL soil, in which the numbers of CFUs comprised only 4.7% of the total number of the control isolates, but this number sharply increased under zinc impact – their percent reached 98.5% of all isolated fungi at 0.2 M $ZnSO_4$ concentration in the medium. Fungal composition of the CFPS was surprisingly significantly distinguishing by the absence of entomopathogenic fungi in the control and Zn-amended plate cultures (Fig. 3).

During the current study, copper also induced some dominance of the entomopathogenic fungi, but in lower degree compared to zinc (Fig. 4).

The highest percentage of the entomopathogenic fungi was determined for DFSS (82.8% of the total number of CFU) under the impact of 3 mM $CuSO_4$. It is noteworthy that, as in the treatment with Zn-amended media, entomopathogenic fungi were not isolated at low (0.25 mM) copper sulphate concentrations. However, at elevated copper concentration in the medium (at interval from 0.5 mM to 3.0 mM) the portion of the entomopathogens in the total number of Cu-resistant isolates increased up to 75.2%. A low percentage of the isolated from ASL and ALC soils entomopathogenic fungi in the control medium also increased under copper sulphate impact.



ASL – arable sandy loam, **ALC** – arable loam-clay, **DFSS** – deciduous forest sandy soil, **CFPS** – coniferous forest peaty soil

Fig. 4. Percentage of the CFU of entomopathogenic fungi in the total number of Cu-tolerant isolates at increasing copper sulphate concentrations in Czapek's agar medium.

DISCUSSION

Some recent investigations are concerned with determination impacts of heavy metals (HM) on soil fungi communities (e. g. Niyazova et al., 1982; Nondgren et al., 1985; Yamamoto et al., 1985; Pennanen et al., 1996a, b; Rajapaksha et al., 2004; Rajapaksha, 2011; Bååth et al., 1998;

Zafar et al., 2007; Macdonald et al., 2011) and only few are related with the distribution of entomopathogenic fungi in HM polluted soil or their resistance to HM impact in a laboratory experiment (Arnebrant et al., 1987; Bååth, 1991; Barker, Barker, 1998; Ropek, Para, 2002; Keller et al., 2003; Tkaczuk, 2003, 2005; Quesada-Moraga et al., 2007). Recently, the majority of studies investigating soil fungus communities have been conducted by molecular or biochemical methods. In most of the studies, ecologists used to analyse phospholipid fatty acids (PLFAs), direct RT-PCR amplification of 18S rRNA molecules and bioavailable restriction fragments length polymorphism (T-RFLPs) of PCR-amplified 18S rinosomal DNA for the detection of fungi to characterise the structure of soil fungus community (Blackwood et al., 2003; Anderson, Cairney, 2004; Anderson, Parkin, 2007; Anderson et al., 2008; Macdonald et al., 2011). However, the drawback of these approaches for fungi is that 18S rRNA sequences often do not provide sufficient taxonomic resolution allowing identification of taxa in mixed communities to genus or species level (Kelly et al., 2003; Anderson, Parkin, 2007; Anderson, Cairney, 2004; Anderson et al., 2008). Anderson et al. (2008) also referred that the combined approach of using DGGE and cloning / sequencing to examine differences in soil fungal communities was not proved a useful method primarily due to the similarities in DGGE profiles between different samples and the fact that the identification of many of the sequenced bands remained unresolved because of their relatively low match with reference database sequences. M-TRFLP method has the ability to analyse several groups of microbial taxa simultaneously (Singh et al., 2006) and has recently been used to investigate the effects of Zn and Cu rich sludge on various groups of soil microbial taxa (Anderson, Parkin, 2008; Macdonald et al., 2011). As it has been referred, the observed decrease in 16:1 ω5c in the zinc-amended soils may reflect a part of the arbuscular mycorrhizal fungi to elevated heavy metal concentrations (Kelly et al., 1999), however, there is also evidence that 16:1 ω5c can be found in some groups of bacteria (Olsson et al., 1997).

We concentrated on a simpler but more time-consuming method – soil fungi community analysis by dilution plate method. This paper reports on laboratory studies of the effect of heavy metals (Cu and Zn) added in the medium on the cultivable fungi from different soil types. Most of the soil fungi belonging to classes Zygomycetes and Deuteromycetes are very common in various agricultural and forest soils (Domsch et al., 2007). The soil dilution plate method used in the current study is a suitable method for isolating fungi of these two classes as it supports releasing and mixing of numerous spores produced by the fungi into the diluted soil suspensions. A reason that may explain why only four fungi of the class Ascomycetes and only one fungus of the class Basidiomycetes were obtained in the present study is that the suitable methods for their isolation were not used, namely, soil-streaming for Ascomycetes isolation (Warcup, 1951) or washing of organic particles from soil and plating on a medium with lignin, quaiacol, and benomyl, which reduce mould growth and allow detection of Basidiomycetes (Thorn et al., 1996). The list of soil fungi in Table 3 may be an underestimation of the total number of species in the soil of four types, because only one isolation method was used. However, soil dilution plate method is the simplest, as well as the most judicious, method by which soil fungi assemblages may be screened (Keller, Bidocha, 1998).

Results of the laboratory experiment also cannot reflect the actual affects of the elevated Cu and Zn concentrations on the fungal communities under field conditions, however, some predictions can be available. Reactions of the fungi to Zn and Cu impact as well as changes in soil fungus communities depending on metal pollution must be interesting to mycologist. Soil fungi represent one of the essential components of the biotic system in natural forests, pastures, grassland and agriculture fields where they are key players in nutrient turnover (Hackl et al., 2005). Entomopathogenic fungi are a separate group, playing a key role in regulation of insect populations, particularly soil-dwelling insect pests (Keller, Zimmermann, 1989; Jackson et al., 2000). Many species belonging to *Hypocreales* (*Ascomycota*) inhabit the soil for a significant part of their life when they are outside of their insect host. Soil is a net sink for many pollutants (such as heavy metals), and the functioning of the overall-terrestrial ecosystem is dependent on soil processes (Tscherko et al., 2007). In Lithuania, the content of Zn and Cu in the land soils

remains at a background level and only in some areas the content of these most harmful soil pollutants, arising mainly from repeated applications of sewage sludge, municipal wastes and animal slurries, and impurities in fertilizers are recorded as elevated as compared with their content in the background (Kadūnas et al., 1999). Results of the present investigation (Table 1) have revealed that none of the soil samples from 12 studied sites had zinc and copper contents exceeding the background concentrations and permissible for our country concentrations (Lithuanian Standard of Hygiene HN60: 2004). It was not expected to obtain statistically significant correlations between the abundance of soil fungi (or species diversity) and the contents of Zn and Cu in the soil studied. No significant correlation between fungal parameters and metal (Zn or Cu) content in soil, as well as their correlation versus the main soil chemical parameters (not presented) were obtained. On the other hand, it is known that both taxonomic and functional diversities of the soil fungi are considered to be highly dependent on water availability, temperature regime, organic matter content, and is closely associated with distribution of plant litter (Zak et al., 2003; Grishkan, Nevo, 2010). The results presented by Zak et al. (2003) demonstrated that plant diversity impacted ecosystem processes by modifying the composition and function of heterotrophic fungal communities in soil. Marfenina et al. (2001) indicated that cultural layers of soils showed higher fungus species diversity and more diverse morphology in comparison with surrounding soils. In the present study, the arable fields (ASL and ALC) were after the vegetative growth of the winter wheat, while forest areas were occupied by deciduous forest stands with dominant tree species *Fraxinus excelsior* L., *Populus tremula* L., *Betula pendula* Roth., a few stands of *Picea abies* L. H. Karst, *Pinus sylvestris* L., *Quercus robur* L, and major species of bushes: *Juniperus communis* L., *Frangula alnus* Mill., *Sorbus aucuparia* L., and *Padus avium* Mill. The second, peaty forest site, was occupied by Norway spruce (*Picea abies* [L.] Karst.); mosses and lichens comprised the ground layer. A suggestion could be that in the present investigation basic soil parameters as well as vegetation were more essential factors influencing soil fungal communities. Forest stand, underground vegetation as well as plant rotation could influence the composition of soil communities in a specific way (Grishkan, Nevo, 2010).

Despite the absence of the correlations between the fungal parameters, different reactions of the fungi from different soil types have been noticeable during the present investigation. Soil fungal communities are comprised of species with different degree of tolerance to any environmental stress (Rajapaksha, 2011). Elevated heavy metal concentrations in soil are one of such stress factors exerting a selection pressure on soil microorganisms (Gadd, 1993; Bååth et al., 1998; Pnnanen et al., 1996a, b; Zafar et al., 2007). Both Zn and Cu are essential elements, although an excess of these metals is toxic for cells and causes metabolic damage: Zn may be less toxic but generally in soils it is present in higher concentrations (Gadd, 1993). The affect of Cu is hundredfold higher than the inhibiting effect of Zn (Barajas-Aceves et al., 1999). Heavy metal affects on soil fungal communities depend on the area management. The agricultural management practices lead to changes in soil pH, which may have the greatest effect on soil biology and functions important to plant and ecosystem health as well as on soil fungus community structure. In Lithuania, acidification of agricultural soils is prevalent and characteristic across the country (Buivydaitė, Vaičys, 1996). It has been noted that phosphorous fertilizers produced from apatite (or from phosphate) introduce a particularly abundant association of chemical compounds (among them Zn and Cu) as soil admixtures (Todorova, Dombalov, 1995). Fuller (1977) in discussing the relatively high mobility of heavy metals with regard to pH considered that in acid soils (pH 4.2–6.6) Zn is highly mobile and Cu practically immobile; in neutral to alkaline (pH 6.7–7.8), Zn is moderately mobile. Abundance of cultivable fungi in the soil of the present study negatively correlated with soil pH, however, correlations were not significant (r_s = –0.8 at P = 0.2). During the present investigation, the pH in soils studied varied from 3.8 to 6.3 (Table 1). Out of 72 soil samples analysed, only eighteen (all from CFPS) showed pH values below 3.8. The highest organic matter content (~45.3%) was in the CFPS, however, traditionally in peaty soil pH is low (in our case 3.8). The complex of those two parameters induced the development of a different fungal community than in other soils studied (Table 4). Positive correlation between the

OMC and contents of Zn and Cu in the soil was determined (r_s = 0.78, at $P < 0.04$), however, none of these soil parameters statistically significantly correlated with the abundance and composition of fungal populations of cultivable fungi, as well as with the Zn- or Cu-tolerant fungal complexes.

During this study all metal-tolerance experiments were done under the laboratory conditions investigating complexes of cultivable fungi able to tolerate elevated concentrations of Cu and Zn in the nutritional medium. To obtain more comprehensive data, we selected different land areas varying in the soil texture, structure and management (Table 1). Firstly, we tried to evaluate the effects of Cu and Zn on soil fungi by the tolerance level of the community in the laboratory experiment. The tolerance level (CL_{50}) is the heavy metal concentration in growth media where a fungal colony forming unit (CFU) decreases to 50% of that in metal unsupplemented media. A low CL_{50} value of a fungal community means that metal-sensitive fungi are dominant and that the community is affected at low metal levels. In the present study, this method has an advantage – it is uniformly adaptable to a variety of soil fungal communities which are examined under the same conditions. Moreno et al. (2001) and Renella et al. (2003) also recommended usage of the ecological dose (ED_{50}) of metals in soil habitat as a possibility to quantify microbial-mediated processes in soil. Although CL_{50} values of Cu and Zn for fungal tolerance in literature are limited, some discussion can be developed. In the present study CL_{50} values of Cu and Zn were comparable for all soil types studied (Table 4) despite of different abundance of fungi in four types of soil (Fig. 1). Although the total numbers of isolated fungi and the numbers of metal-resistant fungi did not statistically correlate with the contents of Cu and Zn in the soil, the level of tolerance in accordance with species composition was different depending on the soil type. Fungal populations from different soil types developing at semi-lethal Cu and Zn sulphate salt doses (2.26 mM and 0.126 M, respectively) were analysed to disclose the complexes of metal-tolerant fungi. A large fraction of cultivable fungi was able to tolerate 0.89 mM Cu^{2+} (~57 mg Cu^{2+} kg^{-1} soil) and 0.05 M Zn^{2+} (~3 270 mg Zn^{2+} kg^{-1} soil) (Table 2) concentrations in the agar media, which are several fold higher than the highest total Cu (11.4 mg kg^{-1}) and Zn (39.9 mg kg^{-1}) content found in the soils studied (Table 1). Far higher copper concentrations such as 300 mg l^{-1} Cu (~5 mM) and less concentrations of Zn – 450 mg l^{-1} Zn (~7 mM) in the culture medium were tolerated by the soil fungi during a study conducted by Rajapaksha (2011); these metal concentrations were also several fold higher than the highest total Cu (101 mg kg^{-1}) and Zn (169 mg kg^{-1}) found in the soils investigated. Huysman et al. (1994) suggested that neither metal tolerant populations nor determined Cu CL_{20} for soil fungi can correlate with the extractable Cu fractions implying that the *in situ* metal concentrations are below the threshold level that would induce metal tolerance in the fungal community. In their study, the diversity of cultivable fungi also indicated that there was no difference in the predominant metal tolerant fungi between virgin and cultivated soils in contrast to the findings of Yamamoto et al. (1985). Huysman et al. (1994) suggested that total Cu content of 30 mg g^{-1} soil was not high enough to induce metal tolerance in fungi. With reference to Rajapaksha (2011), five soils studied by Huysman et al. (1994) which contained DTPA-extractable Cu concentrations in the range of 36–57 mg Cu kg^{-1} soil may have increased the metal tolerance in fungi to some extent though these changes were not reflected in the CL_{20} values analysed. Although the concentration of Zn used in this study was higher than that in the study conducted by Rajapaksha (2011), and the concentration of Cu was lower than used by Rajapaksha (2011) and comparable with that used in the study conducted by Huysman et al. (1994) the main trends of the soil fungi reactions to Zn and Cu stress were comparable. El-Sharouny et al. (1988) amended soil with zinc and copper and determined changes of the soil fungi composition on Czapek's glucose agar at intervals up to 15 weeks. The authors suggest that when heavy metals were incorporated into the isolation medium, they depressed the total count of fungi and affected individual species. During the present study we observed comparable reactions of soil fungi. Most studies were related with the effect of heavy metals on soil fungi in field conditions (e. g. Bååth, 1989; Joradan. Lechevalier, 1975; Niyazova et al., 1982; Nordgren et al., 1985; Huysman et al., 1994; Kelly et al., 1999; Jabbour, Barbercheck, 2009; Kelly et al., 2003). However only a few of them

studied the affect of Zn or Cu on soil fungi when metals are incorporated into the isolation medium (El-Sharouny et al. 1988; Rajapaksha, 2011), therefore, it is very complicated to discuss comparable results and reactions of soil fungal populations in the laboratory experiment. During the study of El-Sharouny et al. (1988), Zn additions (at 10, 50 and 100 ppm concentrations) into Czapek's agar medium decreased fungal counts from 149.6 per mg dry soil in control to 87.2, 49.6 and 12.8 per mg dry soil, respectively. During the present study, low zinc concentrations in Czapek's medium (0.01–0.045 M) stimulated some fungal species thus elevating their total counts in cultivable populations. It is noteworthy that these concentrations are essential for the growth of some fungi. In the study of El-Sharouny et al. (1988), *Fusarium* spp. and *Myrothecium* spp. were more resistant species, however in the present experiment, species belonging to *Isaria*, *Metarhizium*, and *Verticillium* genera dominated in Zn-resistant viable fungal populations. Jordan and Lechevalier (1975) recorded a decrease in the abundance of isolates from genera *Penicillium*, *Oidiodendron*, *Mortierella* and *Paecilomyces* (syn. *Isaria, Spicaria*) near a zinc smelter. Freedman and Hutchinson (1980) found no effect of these heavy metals on the microbial communities. In contrast, El-Sharouny et al. (1988) determined that copper sulphate added into Czapek's agar medium was toxic at all levels and reduced the counts of most fungal species, with the exception of *Aspergillus niger*. Low Cu concentration increased the counts of some fungus species (such as *A. niger*, *A. ustus*, *A. terreus* and *Paecilomyces inflatus*) as compared with control.

It was observed that during the cultivation of fungi on the media amended with copper or zinc a longer lag period was required for the development of metal tolerance. The degree of metal tolerance depended on fungus species and their isolates. In metal-amended media, some fungal isolates started their growth on the first day of cultivation, others – only after 57 days. Another question arising from the data of the present investigation is that in some portion of the soil fungi could grow better at higher heavy metal concentrations than at lower (results not presented). Comparable results were obtained by Yamamoto et al. (1985) in the study of Cu-impact to the soil fungus community; exposure to 1 600 μg Cu g^{-1} soil resulted in tolerance development for 1 000 μg Cu l^{-1} within a week but for concentration ranging from 10 to 100 μg Cu l^{-1} it took about five months. Particular reactions of different saprotrophic fungal groups (saprothrops and pathogens) were observed in the present investigation. There are fungi that have an intrinsically high resistance against heavy metals or that give rise to mutants capable of tolerating high metal concentrations (Zimmerman and Wolf, 2002). These strains, however, often show poor sorption properties since they accumulate far lower amounts of metal than sensitive strains. Apparently the most common entomopathogenic fungi which dominated metal-resistant fungal populations during the present investigation act by this principle: they do not accumulate metals in their biomass but rather bind them outside the cells. Accumulation of copper sulphate and formation of zinc crystals in the nutrient medium with metals were observed during the present investigation (data not presented) and are documented by Fomina et al. (2005): *Beauveria caledonica* fromed oxalate crystals with Cd^{2+}, Cu^{2+}, Zn^{2+} and Pb^{2+} ions. On the other hand, fungal species known as metal accumulators (species belonging to genera *Rhizopus*, *Mucor*, *Aspergillus* as well as *Trichoderma*) were eliminated from the soil fungi populations at low Cu or Zn concentrations in the laboratory experiment of this study.

CONCLUSIONS

In summary, changes in copper and zinc sulphate concentrations in the nutrient media had an effect on the abundance and diversity of cultivable soil fungi in the laboratory experiment. It was shown that zinc and copper influenced fungal populations differently, apparently due to the fact that each of them exerts selection pressure for soil fungi with specific properties. Intermediate concentrations of these metals seem to enhance abundance and diversity of fungal populations. At elevated metal concentrations in the medium the abundance of cultivable fungi decreased with marked elimination of some fungus species from populations. The reactions of the fungal communities from different soil types suggested that there is no general resistance of the total community but rather resistance of particular species. Furthermore, entomopathogenic fungi distinguished by their

tolerance to Zn and Cu even at high concentrations of metals in the medium, at which most species of the cultivable on the control medium fungi were inhibited completely. Among the Zn- and Cu-tolerant fungi dominated common soil entomopathogens *Beauveria bassiana*, *Lecanicillium lecanii*, *Metarhizium anisopliae*, *Isaria farinosa* (syn. *Paecilomyces farinosus*), and *I. fumosorosea* (syn. *P. fumosoroseus*). Some other species such as *Fusarium solani*, *Clonostahys rosea* f. *rosea* and *Aspergillus fumigatus* which recently were proved as insect pathogens were also metal-resistant. Such metal-resistance peculiarities of entomopathogenic fungi may be explored for the search of virulent strains to be applied as biopesticides. We presume that these results can also reflect tolerance of the fungal communities and populations in the soil.

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Dalė Pečiulytė, Vaidilutė Dirginčiutė-Volodkienė

CINKO IR VARIO POVEIKIS KULTIVUOJAMŲ DIRVOŽEMIO MIKROMICETŲ POPULIACIJOMS AKCENTUOJANT ENTOMOPATOGENIŠKUS MIKROMICETUS

S a n t r a u k a

Vario ir cinko poveikį dirvožemio mikromicetų populiacijoms tyrėme laboratorijos sąlygomis. Skirtingo dirvožemio (ariamo smėlingo, ariamo priemolio; miško smėlingo ir miško durpės) pavyzdžius surinkome Vilniaus rajono vietovėse. Metalų poveikio išsamesnius tyrimus vykdėme mikromicetų išskyrimui ir auginimui skirtą terpę (Čapeko mitybinį agarą) praturtindami įvairiais metalų druskų ($CuSO_4$ ir $ZnSO_4$) kiekiais ir įvertindami išaugusių mikromicetų gausą bei rūšių sudėtį. Zn ir Cu druskas, sterilizuotas autoklave, įterpėme į sterilią mitybos terpę. Cinko koncentraciją terpėje keitėme nuo 0,05 iki 0,20 M (0,05 M intervalu), vario – nuo 0,5 iki 3,0 mM (0,15 mM intervalu). Terpėse, praturtintose didesniu metalų druskų kiekiu, mikromicetų gausa pasėliuose sumažėjo, palyginti su kontrolinės (be metalų priedų) terpės pasėliais, o kai kurių bandymo variantų rūšys neaugo. Nepaisant skirtingų mikromicetų bendrijų keturiuose tirtuose dirvožemiuose, varis stipriau slopino kultivuojamus mikromicetus nei cinkas, tačiau abiejų metalų poveikis mikromicetų rūšių įvairovei pasėliuose buvo panašus. Atspariausi tirtiems metalams buvo entomopatogeniški mikromicetai (*Beauveria bassiana*, *Metarhizium anisopliae*, *Lecanicillium lecanii* ir *Isaria* genties rūšys), kurių kamienai skirtingos metalų koncentracijos terpėse vyravo sudarydami iki 90 % visų išskirtų mikromicetų.

Raktažodžiai: kultivuojami mikromicetai, entomopatogeniniai mikromicetai, atsparumas metalams, cinkas, varis