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Original Research

Functioning of Ectomycorrhizae and Soil Microfungi in Deciduous Forests Situated Along a Pollution Gradient Next to a Fertilizer Factory

D. Stankevičienė*, D. Pečiulytė

Institute of Botany, Žaliųjų ežerų 49, LT-2021 Vilnius, Lithuania

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Abstract

Ectomycorrhizae (ECM) and soil microfungi were studied in soil cores from seven unequally polluted forest plots spaced at different distances from a fertilizer factory in Lithuania. The abundance of ECM roots and soil microfungi was visibly different in separate investigation plots. Average amount of ECM root tips during the investigation period (2000-2002) in different forests was from 134 to 1017 tips /100 cm^3 of soil and the length of ECM roots was from 12.2 to 79.8 cm/100 cm^3. The concentration of viable soil fungi revealed during the investigation varied from 1.5 to 566.6 thousands CFU/ g d.w. soil. The forest farthest from the factory exhibited the highest abundance of ECM and diversity of ectomycorrhizal morphotypes, while the abundance of soil microfungi was lowest. The lowest diversity of ECM morphotypes was determined in forests characterized by the highest concentration of heavy metals, nutrients (nitrogen, phosphorus) and the highest microfungal abundance was in forests with the highest concentration of nutrients.

Keywords: ectomycorrhizae, soil microfungi, deciduous forests, pollution

Introduction

Soil mycobiota is quite variable and the presence and distribution of organisms differs with physical and chemical properties of soil (texture, minerals, plants, organic matter, water potential, pH and other). Human impact has been observed to alter the fungal community structure and ectomycorrhizal colonization. A species-specific decrease in ectomycorrhizal fungi, reduction of short roots [1] and declining ectomycorrhizal diversity were attributed to chronic deposition of N and acid [2-5]. Soil microorganisms, mycorrhizal fungi and fungi antagonistic to pathogens are significant in the protection of plants against the effects of pollutants, e.g. toxic metals [6]. Heavy metals might damage the development of ectomycorrhizae [7, 8] and soil microfungi as they can greatly suppress their numbers [9-11]. It is preconditioned by several factors, such as the granulometric composition of soil, quantity and quality of organic matter, pH, total exchange capacity, nutrient availability [9, 12, 13] and finally species-specific characteristics of fungal organism. For example, Tam [14] noted high tolerance of *Pisolithus tinctorius* to some metals (Al, Fe, Cu, Zn, Cd, Cr) in comparison to two strains of *Thelephora terrestris* and *Cenococcum geophilum.* Fungi are involved in bio-geochemical processes of both degradation and synthesis in soil. They adapt to changes by evolving strategies to maintain low intracellular concentrations of toxic pollutants. There is no doubt that firm relations among microbial communities, their activities, and ecologically important processes, such as mineralization and transformation of organic matter exist [15]. Because of the ecological significance of fungi, these parameters should be considered as potentially useful in assessing soil quality. Brokes [16] indicated that such parameters as the number, weight and activity of mi-

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*Corresponding author; e-mail: mikods@botanika.lt croorganisms can be good indicators of soil contamination with heavy metals. Gaseous emissions (SO_2, NO_x) and fall-out of particles enriched in Pb, Zn, Cu and Cd cause acid precipitation and heavy metal contamination at more than 30 km downwind from the smelter [17]. Therefore, pollution of the environment, caused by human impact, could alter the state and diversity of macro- and microfungi along with forest productivity and regeneration [18].

The aim of this study was to investigate the impact of long-term industrial pollution (enlarged amount of nutrients, heavy metals) on the abundance and diversity of ectomycorrhizal roots and soil microfungi in deciduous forests situated at different distances from the chemical factory in Lithuania. The first product of this chemical enterprise was obtained in 1963. At present, the major product of the company is nitrogen phosphorus fertilizer. The main emission products are NO_x, CO, sulphur anhydrite, apatite and fluorine. Hard pollutants emitted from the chimneys make up about 140 t/year (data provided by the Ministry of the Environment of the Republic of Lithuania).

Material and Methods

Study Site

The study site was located near the city of Kėdainiai in Central Lithuania (70 m above sea level). The average annual precipitation and average air temperature were 550 mm and 6.4° C. Seven 30x30 m study plots situated at different distances from the pollution source were chosen. Care was taken to choose plots with similar vegetation along the gradient of pollution. The study area was occupied by deciduous forest stands. Dominant tree species were *Fraxinus excelsior* L., which is known to form vesicular–arbuscular mycorrhizae, *Populus tremula* L.*, Padus avium* Mill.*, Corylus avellana* L., which form ectomycorrhizae. The nearest plots Juodkiškis (“2” – 55°16’ N, 24°01’ E) and Zabieliškis-Šilainėliai (“1”- 55°14’ N, 24°01’ E) were at a distance of about 0.7-3 km in the eastern and southeastern directions, accordingly, Vilainiai (“3” – 55°18’ N, 24°01’ E) and Pašiliai (“7” – 55°03’ N, 23°58’ E) forests – about 4 km in northeastern and 5 km southwestern directions, Berunkiškis (“6” – 55°13’ N, 24°08’ E) and Stebuliai (“4” - 55°19’ N, 24°06’ E) – about 8 km in southeastern and 9 km northeastern, and Lančiūnava (“5” – 55°20’ N, 24°12’ E) forest – about 15 km in the eastern direction from the emission source.

Sampling and Observation of Ectomycorrhizae

The chosen forest plots were visited twice a year - in the beginning of May and October during 2000–2002. The state of ectomycorrhizae (ECM) was determined in soil cores of 4.5 cm diameter and 7 cm depth. Three representative samples for each research plot were composed of 18-20 randomly taken soil cores. Soil samples were stored at 4° C until further ECM investigation. Ectomycorrhizal roots were processed within two weeks after the collection of soil cores. For investigation of ectomycorrhizae, from each representative sample 100 g of soil were taken (another part of soil was used for chemical analyses). The soil sample was soaked in tap water overnight and the roots gently cleaned under running water using the 0.5 mm sieve. The following criteria were chosen for evaluation of the ECM state:

1. the number of ectomycorrhizal root tips in 100 cm^3 of soil,
2. the length of ectomycorrhizal roots in 100 cm^3 of soil,
3. diversity of ectomycorrhizal morfotypes in the investigation territory.

ECM roots were sorted under stereoscopic microscope (MBS-10) and divided into dead (wrinkled or shrunk, without turgor) and living. Living ECM was analyzed under a Janoval Carlzeiss Jena microscope and sorted into morphotypes using the criteria of Agerer [19]: hyphal anatomy and colour, rhizomorph characteristics, mantel colour and structure, branching patterns. The number of tips belonging to separate morphological types was counted.

Sampling and Observation of Soil Microfungi

Three representative samples for each research plot were composed of 18-20 random soil samples from the top 7 cm after removal of litter. Microfungi were analyzed by cultivation – viable count. Viable counts were determined by plating 1 ml of 10-fold soil suspension dilutions (five replicates) on malt extract agar (MEA 75, Oxoid). Streptomycin (40 µl/l) was added to this medium for inhibition of bacteria. Inoculated dishes were incubated up to seven days at 25ºC in the dark. After 3–7 days incubation numbers of viable propagules of microfungi were counted. Concentrations of viable fungi in soil were expressed as colony-forming units (CFU) in g of dry weight (d.w.) soil.

Soil Analyses

The concentration of nitrogen and phosphorus was determined photometrically applying the photometer “SPEKOL11”, potassium by applying flame photometer “FLAPHO41”, and content of humus colorimetrically [20]. Soil pH_{KCL} was measured potentiometrically with a glass electrode in a 1.0 M KCl suspension. Concentrations of heavy metals – Pb, Cd, Zn, Cu, Cr, Ni and As – in soil were determined using a Perkin-Elmer Zeeman 3030 atomic absorption spectrophotometer. Content of organic matter (OMC) was calculated as the percent of loss-on-ignition (at 550°C for a minimum of 3 h) from soil dry matter [21].

Data analysis was carried out employing the methods of statistics [22] using *Stattistica 4.5* software.

Table 1. Chemical composition of soil (dw) from the investigated forest plots (1-7).

Plots (distance from the factory, km)	N (%)	P (%)	K (mg/kg)	Humus (%)	pH (KCl)
2 (0.7 km)	0.86± 0.45	0.106±0.024	133.02±68.23	9.89±3.46	4.01±0.2
1 (3 km)	0.39± 0.05	0.052±0.015	143.3±35.61	6.23±1.06	4.66±0.23
3 (4 km)	0.42± 0.08	0.049±0.01	117.7±31.12	7.03±0.98	6.02±0.83
7 (5 km)	0.74±0.09	0.076±0.019	146.07±52.46	9.06±1.72	5.27±0.11
6 (8 km)	0.25±0.03	0.033±0.012	129.05±42.77	4.57±1.02	4.85±0.35
4 (9 km)	0.43±0.06	0.051±0.014	105.47±22.78	6.76±0.67	6.09±0.15
5 (15 km)	0.41±0.08	0.051±0.022	109.7±39.87	6.75±0.93	5.03±0.82

Table 2. Concentration of heavy metals (μgg^{-1} dw) in soil of investigated forest plots (1-7).

Plots	Pb	Cd	Ni	Cr	Cu	Zn	As	sum
2	13	0.14	6	10.5	6.5	16	0.8	52.94
1	20.5	0.12	5.7	12.5	4.82	19.5	1.2	64.34
3	12.5	0.16	6.5	14.5	5.8	23	1.6	64.06
7	11.2	0.24	11.5	21	8.5	25	1.3	78.74
4	8.5	0.06	5.6	11.5	3.6	16	1.02	46.28
6	10.4	0.12	5.7	12	4.35	18.5	1.1	52.17
5	10.5	0.17	6.4	13	4.82	18	1.25	54.14

Results and Discusion

Chemical Characteristics of Forest Soil

The highest concentration of nutrients (N, P, K) was determined in the 2nd and 7th investigation plots distanced 0.7 and 5 km, respectively, from the factory (Table 1). The lowest amount of nitrogen and phosphorus was found in the 6th plot and of potassium in the 4th and 5th plots, which were distanced 8 km, 9 km and 15 km, respectively. Negative correlation was observed between potassium concentration in the forest soil and distance from the pollution source. Concentration of nitrogen and phosphorus positively correlated with concentration of humus maximum value, which was determined in forests at a distance of 0.7 and 5 km. The lowest value of pH was determined in the soil of forests situated closest to the factory. Correlation between pH and the distance from the factory was not significant

The highest concentration of heavy metals was determined in soil of the 7th plot situated 5 km from the factory, and in soil of the 1st and 3rd (3 and 4 km respectively) plots and the lowest – in soil of the 6th plot (8 km) (Table 2). Forests situated at a distance of 0.7, 9 and 15 km (2nd, 4th and 5th) took the intermediate position. Evaluation of the distribution of various metals in different investigated forests revealed a negative correlation between the distance and concentrations of Pb, Cu (R = -0.82 and -0.52 respectively). Thus, forests situated at a distance of about 3-5 km from the factory were the most polluted by heavy metals. The highest concentrations of Zn, As, Cr and Ni distinguished these forests.

Abundance and Diversity of Ectomycorrhizae

Abundance of ectomycorrhizal roots was evidently different in separate investigation plots ($P < 0.05$). Average amount of ECM root tips during the investigation period in different forests varied from 134 to 1017 tips/100 cm^3 of soil and the length of ECM roots was from 12.2 to 79.8 $cm/100cm^3$ (Table 3). These parameters positively correlated with the distance from pollution source (R=0.64 for the number of tips and R=0.68 for the length of ECM roots) (Fig. 1). It is noteworthy that the lowest amount of ECM roots was determined in the forests spaced at a distance of about 3-5 km from the factory where the number of tips in separate plot made 4-7% of the total amount in the investigated area. The most intensive process of ECM formation was in the forest situated at the farthest distance, i.e., about 15 km from the pollution source, where relative abundance of ECM tips was 29% of the total amount in the investigated area. Analysis of soil chemical composition showed that the lowest concentrations of nutrients and heavy metals were registered

Table 3. Abundance of ECM and microfungi in different investigation plots (average of investigations in 2000-2002).

Plot (distance from the factory, km)	A	SD	B	SD	C	SD
2 (0.7)	417	329	29	12.1	263.6	90.1
1 (3)	250	95	23.2	11.7	215.1	57.4
3 (4)	135	58	10.1	4.8	110.2	31.1
7 (5)	134	85	12.2	8.4	125.1	36.9
6 (8)	642	424	48.5	26	87.2	27.9
4 (9)	892	357	58.9	28.2	93.4	30.8
5 (15)	1017	377	79.8	30.2	135.7	42.8

A - number of ECM roots tips/100 cm^3 of soil, B - length of ECM roots cm/100 cm^3 of soil, C - concentration of viable microfungi on MEA (thousands CFU/g dw soil), SD - standard deviation

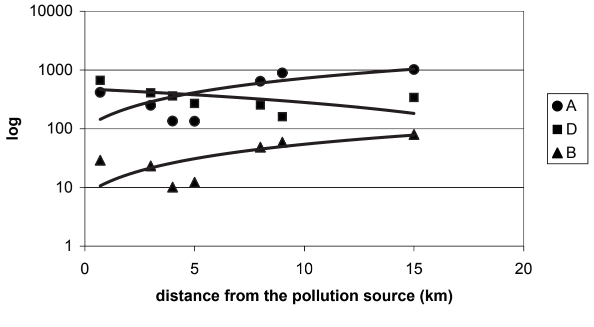

Fig. 1. Relationship between the abundance of ectomycorrhizae (A – number of ectomycorrhizal root tips/100 cm^3 of soil, B – ectomycorrhizal root length cm/100 cm^3 of soil), microfungi (D - thousands CFU/g dw soil) in soil (log-transformed) and the distance from pollution source.

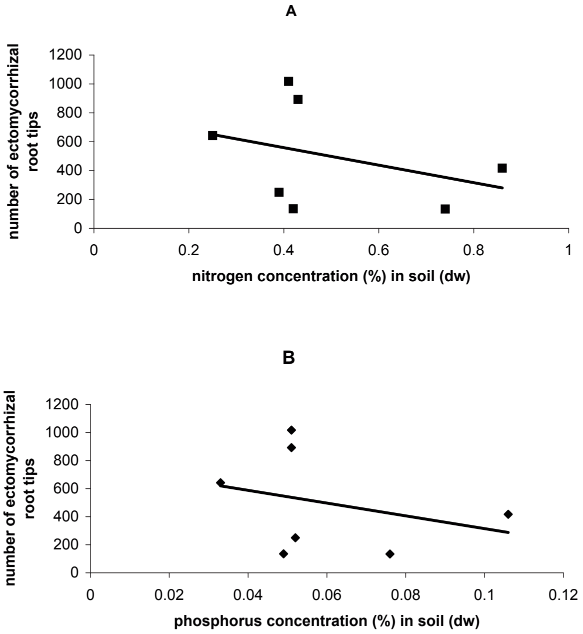

Fig. 2. Relationship between the number of ectomycorrhizal root tips and nitrogen (A), phosphorus (B) concentrations in soil.

in the forests situated at larger distance (8-15 km) from the factory where the abundance of ECM root tips was the highest. Meanwhile, soil of forests spaced at a distance of about 5 km was characterized by the highest amount of pollutants. A significant negative correlation was found between the total amount of investigated heavy metals in soil and the distance from the pollution source (R= -0.78), also between the distance and concentration of potassium (R = -0.75). This is in contrast to the relationship between the distance and the abundance of ECM (Fig. 1). It was determined that the number of infected root tips and fruit body production of ectomycorrhizal fungi negatively correlated with the increased concentration of N in soil [23 – 25]. Baar [26], investigating root growth and ectomycorrhizal development in the natural forest stand on nitrogen-poor soil and in the planted stand on nitrogen-enriched soil revealed that root length, number and frequency of ectomycorrhizal tips were generally higher in natural stand plots than in planted stand plots. The major number of tips in natural stand was 2086/100cm^3 of soil and this parameter was many folds lower in planted stand -141/100 cm^3. It was also shown that ectomycorrhizae formation was reduced by eutrophication in wet alder carr forest [27]. The number of ectomycorrhizal root tips was 117 and 610/100cm^3 of soil in eutrophied and undisturbed peatland stands, accordingly. Our data accords with these results. Lower abundance of ECM root tips was in forests with the highest concentration of nutrients – nitrogen and phosphorus (Fig. 2). Study plots characterized by the lowest development of ectomycorrhizae were distinguished by the highest amount of both nutrient and heavy metal (Fig. 3). The suppressed ability of symbionts to form ectomycorrhizal structures due to heavy metals has also been noted by other authors [28]. Thus, the decreased ECM root tip formation in forests situated near the chemical factory of fertilizers should be associated with the changes in chemical composition of soil specifically with the elevated concentration of nutrients and heavy metals.

The number of ECM morphotypes in different plots positively correlated with the distance from pollution source (R=0.77) same, as the abundance of ECM roots in the soil. The least amount of morphotypes was determined in 1st-3rd and 7th close distance investigation plots (i.e., to 5 km from the factory) where the concentration of heavy metals and nutrients was higher. Morphotypes found in these forests composed from 8 to 11% of the total amount of morphotypes in the investigated territory. Meanwhile in the farthest forest (5th plot, distance – 15 km) determined morphotypes made 27% of their total amount. Thus, the number of morphotypes in the forests spaced at a distance ca. 5 km from the pollution source was about three times lower than in the farthest one.

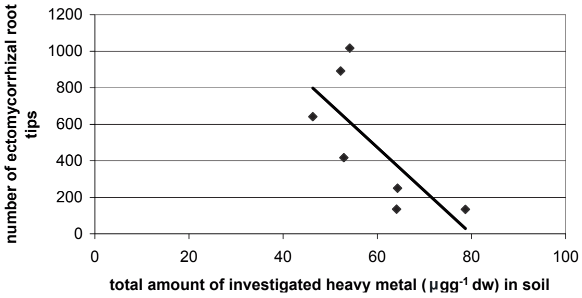

Fig. 3. Relationship between the number of ectomycorrhizal root tips and heavy metals concentration in soil.

This means that increased concentration of heavy metals and N, P in the soil can change both quantitative and qualitative below-ground community structure of ectomycorrhizal fungi. Baxter et al. [25], while investigating oak forest stands exposed to contrasting anthropogenic impacts, found differences in ectomycorrhizal diversity and community structure between the urban and rural stands. They hypothesize that shifts in ECM composition and colonization between urban and rural stands are due to the sensitivity of some ectomycorrhizal fungi to N, heavy metal, etc. deposition. This was especially pronounced for the dominant genera *Cortinarius* and *Russula* [29]. Fungi of the *Lactarius* genus were less affected, whereas fruitbody production and root colonization of some species was more intensive, e.g., *Lactarius rufus* (Scop.: Fr.) Fr., *L. tejogalus* (Bul l.: Fr.) S.F. Gray [30]. Our investigations showed that ectomycorrhizae formed by fungi of the genus *Lactarius* was dominant in forests distinguished by the increased concentration of N and P as well. Ectomycorrhizae, resembling *Cortinarius* according to their morphological and anatomical features, was determined in forests (5th and 6th) with the lowest concentration of nutrients. This is consistent with records [30] of a decreased percentage of roots colonized by various *Cortinarius* species in an N-affected forest. We obtained similar data while analyzing distribution of ECM formed by *Cenococcum geophilum* Fr., which was determined according to morphological and anatomical features of hyphae and ectomycorrhizal mantel [19]. Abundance of this ECM varied distinctly among different investigative plots. The highest amount of ECM tips of this species was found in forests spaced over 9 and 15 km from the factory. This number in other investigation plots comprised only 10% of the total amount of *C. geophilum* ECM found in the investigation territory. These investigations also confirmed previous data [31] that ectomycorrhizae formed by *C. geophilum* was more frequent in less polluted forests and contradicted the suggestions that this morphotype could be ranked among the bioindicators of the pollution of forest soil [32].

Thus, summarizing data of presented ectomycorrhizal investigation, we can conclude that the decreased ECM root tip formation in forests situated near the factory of fertilizers is associated with the changes in chemical composition of soil and specifically with the increased concentration of nutrients and heavy metals which can cause changes in below-ground community structure as well.

Abundance and Diversity of Microfungi

The standard plate count, direct enumeration method used for microfungi, which was used in current investigation, was of fairly limited value. The number and diversity of microfungi in soil can be extremely large, but often only a small proportion (generally <10%) of them is viable or form colonies on agar plates. Many fungi from natural environment do not grow on synthetic media. Populations, which grow on that media, sometimes could be confused with other members of the community [33]. These are termed the viable portion of the fungal community that in current investigation was performed on malt extract agar (MEA) medium. We tried to compare abundance of microfungi in soil samples collected at the investigated forest sites. The number of viable on MEA microfungi during the investigation period varied from 1.5 to 566.6 thousands CFU g^{-1} d.w. soil. Mean counts of the microfungi were the highest in the 1st and 2nd plots and statistically significantly differed ($P < 0.05$) from their concentrations in the other plots (Table 3). The lowest viable on MEA medium number of microfungi was observed in the 6th plot, located 8 km from the pollution source and, perhaps, was due to low organic matter content and the least water content in soil of that forest plot. Statistically significant but low positive correlation ($R=0.57$) between determined microfungal viability by CFU counts on MEA and organic matter content in soil samples was determined. CFU counts of microfungi in forest plots positively correlated with N concentration ($R=0.59$) as well as with Zn ion concentration ($R=0.61$) in soil. Valuable positive correlation of the detected microfungal counts with both total ($R=0.77$) and mobile phosphorus ($R=0.74$) concentrations in the soil was determined. No statistically significant correlation between microfungal counts and

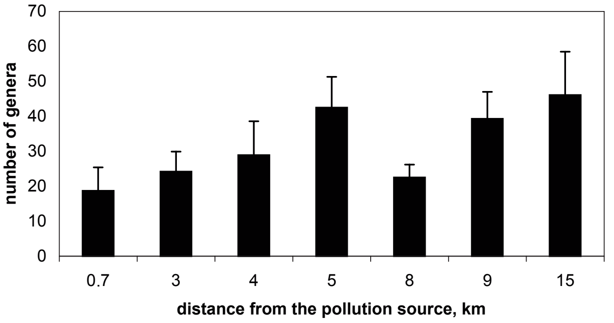

Fig. 4. Distribution of genera of microfungi isolated from the soil of different forest plots spaced at varying distance from pollution source. Mean values (± S.D.) from all soil collections over investigation.

concentrations of other heavy metal was found and low negative (R= -0.59) correlation with the distance from the pollution source was detected (Fig. 1).

Total number of genera of microfungi isolated from the soil of seven plots investigated varied from 18 to 46 (Fig. 4). The highest diversity of microfungus genera was determined in soil samples collected from the 5th and 7th plots, while the lowest in the soil samples collected from the 2nd and 6th plot. *Penicillium* was the most commonly occurring genus among the isolates on MEA medium in all soil samples and was also the largest contributor to the total fungal numbers in all samples except soil samples from the 2nd and 6th plots, where its proportions were similar to those of the *Gliocladium* and *Acremonium* genera. There were also significant differences in the prevalence of the *Absidia, Cladosporium, Mucor,* and *Trichoderma* genera (logistic regression: $P < 0.05$). It must be noted that *Cladosporium* spp. dominated in fungal community of the 2nd plot soil, where the diversity of microfungi was the lowest. Fungal community structure determined during that investigation in the 4th plot was dominated by *Absidia* spp. and *Mucor* spp., and the community isolated from the 5th plot soil was dominated by *Mortierella* spp. and *Gliocladium* spp. With the increasing distance from the pollution source, fungal diversity slightly increased, but fungal community composition in the highest degree depended on the soil characteristics and especially on the total concentration of nutrients and heavy metal ions.

Thus, the data obtained suggested that microfungal abundance was in a negative correlation with the distance from the pollution source, meanwhile, the number of genera increased with the distance from the pollution source. Concentrations of the microfungi were the highest in soil of the 1st and 2nd plots, while the number of genera was the lowest in the soil of those plots. Community of microfungi in the 1st and 2nd plots was dominated by species (*Cladosporium* spp., *Paecilomyces farinosus* (Holmsk. Et Gray) a.H.S. Br. et G.Sm., *Penicillium* spp.) which are known as resistant to pollutants and able to grow on MEA and other media [34]. Microfungal communities were composed of saprophytic, phytopathogenic, and entomopathogenic species. Very common soil microfungi such as *Mucor hiemalis* Wehmer and *Trichoderma viride* Pers. Ex Gray are known as able to improve the mobility of heavy metals in soils [35]. These microfungi were abundant in soil of the 4th, 5th, and 7th plots, where diversity of microfungi was higher. The obtained data showed that microfungal diversity was considerably reduced by heavy metal pollution and dependent on soil solid phase, composed of inorganic and organic materials.

As a conclusion, it is to be stated that microfungi and ECM can act as important evaluation criteria in broad scale soil monitoring due to their intense reaction to pollution-induced chemical soil changes.

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