IMPACT OF VARIOUS FACTORS ON THE DIVERSITY OF SOIL MICROORGANISMS IN AGRICULTURAL AND FOREST LANDS

DOCTORAL THESIS

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Subfield of microbiology

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KOPSAVILKUMS

Pētījuma mērķis bija raksturot augsnes saprofītisko baktēriju un micēlijsēņu populācijas mērenā klimata augsnēs sezonāli mainīgos apstākļos, kā arī atkarībā no dažādiem zemes izmantošanas veidiem (meža zemes, aramzemes, pļavas, bijušās lauksaimniecības augsnes), izmantojot gan metodes, kas balstītas uz mikroorganismu kultivēšanu, gan arī molekulārās metodes, izvirzot hipotēzi, ka augsnes mikroorganismu populāciju struktūra un daudzveidība ir atšķirīga dažādos zemes lietošanas veidos un sezonāli mainīgos klimatiskajos apstākļos, un ka abas metožu grupas dod salīdzināmus rezultātus. Papildus tam augsni no veselām skujkoku audzēm salīdzināja ar augsni no audzēm, kas inficētas ar *Heterobasidion annosum sensu lato*, augsne no Eiropas apšu audzēm tika salīdzināta ar augsni no hibrīdapšu audzēm uz bijušās lauksaimniecības zemēs, kā arī augsni no bioloģiskās lauksaimniecības laukiem salīdzināja ar augsni no konvencionālās lauksaimniecības laukiem.

Pētījumos tika izmantotas vairākas metožu grupas. Pirmkārt, augsnes saprofītisko micēlijsēņu un baktēriju populāciju kvantitatīvā analīze tika veikta, izmantojot klasiskās mikrobioloģijas metodes, t.i., mikroorganismu koloniju veidojošo vienību skaita noteikšana uz dažādām mikrobioloģiskajām barotnēm – barības vielu agara, triptona sojas agara, iesala ekstrakta agara, Bengālijas rozā agara ar hloramfenikolu; mikroskopisko micēlijsēņu izolēšana un identifikācija; mikroskopisko micēlijsēņu daudzveidības noteikšana. Otrkārt, tika izmantotas molekulārās bioloģijas metodes - augsnes kopējās DNS izdalīšana, polimerāzes ķēdes reakcijas, izmantojot universālus baktēriju un sēņu oligonukleotīdus, baktēriju un sēņu daudzveidības noteikšana ar amplificētās rRNS restrikcijas analīzi 2% agarozes vai 6% poliakrilamīda gēlā; qPCR; genomiskās DNS ekstrakcija no izolētajām sēņu tīrkultūrām un identifikācija, izmantojot rRNS ITS1-5.8S-ITS2 reģionu. Identificētās micēlijsēnes tika deponētas Latvijas Mikroorganismu kultūru kolekcijā. Treškārt, tika noteiktas *Penicillium* ģints izolātu antagonistiskās īpašības pret *H. annosum* izolātiem *in vitro*.

Kopumā pētījuma rezultāti atklāja svarīgus aspektus attiecībā uz vides faktoru un zemes apsaimniekošanas prakses ietekmi uz augsnes saprofītisko mikroorganismu populācijām Ziemeļu mērenajā zonā lauksaimniecības un meža zemēs.

ABSTRACT

The aim of study was to characterize populations of soil saprophytic bacteria and fungi in temperate climate soils in seasonally changing conditions, as well as in different land use types (forest lands, arable lands, meadows, former agricultural soils) using the methods that are based both on cultivation of microorganisms as well as molecular analysis. A hypothesis was proposed that soil microbial population structure and diversity is different in various land use types and in seasonally changing conditions, and that both groups of methods give comparable results. In addition, soils from healthy conifer stands were compared to soils from stands infected with *Heterobasidion annosum sensu lato*; soils from European aspen stands were compared to soils of hybrid aspen stands on former agricultural lands, as well as soils used in organic agriculture were compared to soils used in conventional agriculture.

Several groups of methods were used in the investigations. First, quantitative analysis of populations of saprophytic soil fungi and bacteria were performed using classical microbiology methods, i.e., enumeration of microbial colony-forming units on various microbiological media – nutrient agar, tryptic soy agar, malt extract agar, Rose Bengal agar with chloramphenicol; isolation and identification of dominant filamentous fungi; estimation of diversity of cultivable filamentous fungi. Second, methods of molecular biology were used, i.e., extraction of total soil DNA, polymerase chain reaction using universal bacterial and fungal oligonucleotides, estimation of bacterial and fungal diversity with amplified rDNA restriction analysis in 2% agarose or native 6% polyacrylamide gel electrophoresis; qPCR; genomic DNA extraction from fungal isolates in pure cultures and identification using sequencing of rDNA region ITS1-5.8S-ITS2; submission of identified fungi to the Microbial Strain Collection of Latvia. Third, antagonistic properties of isolates from genus *Penicillium* against *H. annosum* isolates *in vitro* were measured.

The results revealed important aspects of soil microbiological ecology in respect to the impact of environmental factors and land management practices on populations of soil microorganisms in agricultural and forest soils of Northern temperate zone.

CONTENTS

Introduction

Owing to United Nations Convention on Biological Diversity, significant attention has been paid to the investigations of biological diversity including the diversity of soil microorganisms.

In general, soil microbial populations are studied fosucing on separate ecological groups – ectomycorrhizal fungi, saprophytic fungi and bacteria, entomopathogenic fungi etc. Microbial diversity has been evaluated in various soils – forest soils (arctic, boreal, tropical rainforest, temperate zone soils) and agricultural soils. Saprophytic microorganisms in Northern temperate zone are comparatively less studied.

Major factors affecting microbial diversity in soil are soil forming processes, physicochemical properties of soil, soil particle size distribution, land use type (forest lands, arable lands, meadows, former agricultural lands) and vegetation as well as such seasonally changing factors as soil moisture content and temperature. In their turn soil saprophytic microbial populations can affect and suppress plant pathogenic microorganisms, for example, root rot fungus *Heterobasidion annosum sensu lato* (*s.l.*).

Bacterial and fungal populations in the soil can be analyzed with methods based on isolation and cultivation of these microorganisms in pure culture. These methods are used for several decades and they are considered to be standard methods for identification and quantification of soil microorganisms. However, there is an increase in number of studies in which soil microbial populations are investigated with molecular methods, taking into account the fact that the largest part of soil microorganisms are not cultivable under laboratory conditions.

The following problems for investigations were nominated, such as characterization of populations of soil saprophytic bacteria and fungi in temperate climate soils in seasonally changing conditions, as well as in different land use types (forest lands, arable lands, meadows, former agricultural soils) using both methods that are based on the cultivation of microorganisms and also molecular methods, proposing a hypothesis that soil microbial populations are different in various land use types and in seasonally changing conditions, and that both group of methods give comparable results. Besides that soils from healthy conifer stands were compared with soils from stands infected with *H. annosum s.l.*, soils from European aspen stands were compared with soils of hybrid aspen stands on former agricultural lands, as well as soils of organic agriculture were compared with soils of conventional agriculture. In all investigations the hypotheses were proposed that soil microbial populations could be significantly different between mentioned soil types.

The aim of the study was to characterize fungal and bacterial populations in various groups of agricultural and forest soils, using classical microbiology methods that are based on cultivation of

microorganisms and molecular biology methods.

To achieve the aim, the following **tasks** were proposed:

1. To use both classical microbiology methods that are based on cultivation of microorganisms as well as molecular biology methods and compare the results;
2. To determine the impact of seasonally changing climatic conditions and other environmental factors on soil microbial populations;
3. To carry out investigations to compare agricultural and forest soils, organic and conventional agriculture soils, soils from stands infected with *H. annosum s.l.* and healthy conifer stands, as well as soils from European aspen and hybrid aspen;
4. To isolate and to identify dominant members of filamentous fungi from analyzed soil samples and determine their antagonistic properties against *H. annosum s.l.* isolates.

1. Theoretical background

Soil is a highly heterogeneous environment and different components of the solid fractions in soil (sand, silt, clay, organic matter etc.) provide variable microhabitats. The major factors affecting microbial diversity in soil are soil forming processes, physicochemical properties of soil, soil particle size distribution, vegetation and land use type (management). Relative impact of these factors differ in different soil types, horizons and climatic zones (Garbeva et al. 2004). Several studies investigating the effect of different plant cultures and soil types have shown, that the soil type has the greatest influence on the structure of microbial populations (Chiarini et al. 1998; Grayston et al. 1998; Buyer et al. 1999; Marschner et al. 2001; Wieland et al. 2001), although under some conditions the influence of the vegetation may prevail (Baudoin et al. 2002). Many publications emphasize the influence of the land use (agricultural lands, grasslands, forests and pastures, etc.) on the structure of the soil microbial communities (Nüsslein and Tiedje 1999; Drijber et al. 2000; McCaig et al. 2001; Webster et al. 2002; Clegg et al. 2003). Agricultural management practices, such as crop rotation, tillage, compost, manure, pesticide and synthetic fertilizer application, and water regime, are key determinants of microbial community structure in agricultural soil. Vegetation is also important factor since plants are providing microorganisms with specific carbon sources (Garbeva et al. 2004).

Forest soils are different from agricultural soils by their vertical stratification due to the forest litter accumulation that is forming humic horizon, with saprotrophic soil fungi being one of the main groups of soil microorganisms responsible for litter degradation (Zeller et al. 2007; Baldrian et al. 2010; Allison and Treseder 2011). Their extracellular enzymes are active not only in the litter layer but also in the deeper horizons (Baldrian et al. 2010). It is known that fungi in the soil constitute a significant part of soil biomass and they have several important functions in the soil, such as decomposition of organic material, nutrient cycling, formation of soil aggregates and mycorrhizal symbiosis (Kabir et al. 2003). Only small part of soil fungi (17%) (Bridge and Spooner 2001) and bacteria (0.1–1%) (Torsvik et al. 1996) are cultivable in laboratory conditions.

It is reported in the literature that several properties of site and soil also affect the occurrence

and severity of the damage of such serious tree root pathogen as *Heterobasidion annosum s.l.* that is widely distributed in Latvia (Grantina et al. 2000) and in other countries of boreo-nemoral region. In general, high risk of *H. annosum* damage has been associated with environmental conditions and climate zones that determine soil properties and soil types. According to investigations in Great Britain (Pratt et al. 2002), *H. annosum* hazard increases with the increase of site fertility (mainly on well drained calcareous soils with high pH value). However, the hazard decreases with the increase of soil moisture (surface water gley and groundwater gley features) and accumulation of peat, but this relationship depends on the climate zone. Soils with gley features in warm and dry climate still have high infection risks in comparison to other climate zones. According to Kaarna-Vuorinen (2000), the relative frequency of root and butt rot is higher on drained peatland sites than on undrained peatland sites. High incidence of root rot correlates with a lack of such antagonistic fungi as *Trichoderma* spp. and *Penicillum* spp. (Korhonen and Stenlid 1998; Stenlid and Redfern 1998). Several *Penicillium* species have been isolated from the surface sterilized roots of healthy-looking Scots pine and Norway spruce seedlings (Ndobe 2012). *Penicillium islandicum* and *Penicillium* sp. have been detected in 40% of *Pinus mugo* roots after fire in the infection centres of root rot (Lygis et al. 2010). *Penicillium adametzii* have shown antagonistic properties against *H. annosum s.s. in vitro*, as well as using chloroform extract of this fungus on infected *Pinus sylvestris* seedlings (Szwajkowska-Michałek et al. 2012). Members of *Penicillium* genus have been detected as natural stem surface colonizers (*P. aurantiogriseum, P. brevicompactum, P. canescens, P. daleae, P. simplicissimum, P. velutinum*) (Varese et al. 1999).

Besides traditional forest tree species such as spruce, pine, birch etc., short rotation energy crops are becoming more popular in the forestry of Latvia and worldwide. The cultivation of short rotation energy crops on former agricultural soils differs from agricultural practices with reduced soil tillage that in long term it can cause changes in the vertical distribution of soil microorganisms – increased microbial biomass in the upper 5 cm of the soil and decreased in the subsoil (Makeschin 1991). Cultivation of poplars and willows introduces ectomycorrhizal fungi in the former agricultural soils that normally contain saprophytic fungi, increasing the diversity of basidiomycetes in general (Lynch and Thorn 2006).

The dominant active populations of soil microorganisms are changing in different seasons of the year. Several studies are available on the impact of seasonal changes upon the soil microorganisms in arctic soils (Nemergut et al. 2005), deserts, tropical soils and other soils in extreme environments (Dion 2008). Comparatively few investigations have been published about microbial populations in the soils of the Northern temperate zone (NTZ) in which climatic conditions during winter are characterized by permanent or temporal snow cover and a significant decrease of topsoil temperature below zero.

Abiotic conditions of soil (pH, moisture content and temperature) affect soil microorganisms in all soil types and land use variations (Jurgensen et al. 1997; Hackl et al. 2004; Setälä and McLean 2004; Borken et al. 2006; Ruisi et al. 2007; Zachow et al. 2009).

Two main approaches may be employed to analyze soil microbial communities – conventional plating of cultivable microorganisms and molecular methods that are independent of cultivation. Both approaches have their advantages and disadvantages. Only a small portion of all microorganisms in soil can be cultivated in standard laboratory conditions, the isolation of pure cultures and lengthy

effort-taking labour is needed for the determination of taxonomic identity of the isolates. In contrast, molecular methods give general insight into genetic heterogeneity of soil microbial communities and allow identifying specific microorganisms without isolation, but they are highly dependent on the success of the isolation of DNA from soil, eventual presence of the DNA amplification or restriction inhibitors, choice of the primers, discriminating power of analysis etc. (Kowalchuk et al. 2006). DNA extraction problems significantly influence also results in soil metagenomics (Feinstein et al. 2009).

Amplified rDNA restriction analysis (ARDRA) gives genetic fingerprinting of simple communities, populations or phylogenetic groups. High resolution discrimination may be obtained at species level. In soil microbiology this method is used to determine diversity within phylogenetic or functional groups of microorganisms (Lynch et al. 2004). Digested PCR products can be run in polyacrylamide (Pérez-de-Mora et al. 2006) or in agarose gel (Smit et al. 1997; Schwieger and Tebbe 2000; Chabrerie et al. 2003). Usually several restriction enzymes are used and obtained fragments can be analyzed as separate (Schwieger and Tebbe 2000; Klamer and Hedlund 2004) or combined (Wang et al. 2008) data sets.

Abundance of the specific groups of microorganisms in soil may be determined by quantitative PCR (qPCR) (Filion et al. 2003; Kabir et al. 2003; Kolb et al. 2003; Smits et al. 2004; Fierer et al. 2005). This method has been employed for quantifying presence of genus *Trichoderma* fungi in soil, estimating their potential to counteract the activity of fungal plant pathogens (Cordier et al. 2007).

The theses presented include six investigations. The first study (I) addressed investigation about the influence of the land use (forest, abandoned former agricultural land, arable land, meadow) upon number and abundance of soil fungi and bacteria and genetical diversity of fungal communities estimated by conventional and molecular methods of analysis. The main aspect of this investigation was the approval of molecular methods for the application in soil microbiology.

The second investigation (II) was about seasonal changes of NTZ spruce (*Picea abies*) forest soil microbial populations. The aim of this work was to analyze seasonal changes of the microbial populations in spruce forest soil from two closely located forest stands with the same dominant tree species, but with different relief, soil and vegetation types using classical microbiology methods and molecular methods.

The rest of the investigations were more specifically focusing on more narrow scientific problems with the same common research subject – soil fungal and bacterial populations. The third investigation (III) was about particular characteristics of soil microbial communities in forest stands infected with *Heterobasidion parviporum* and *Armillaria* spp. The fourth investigation (IV) was about abundance and diversity of soil bacteria and fungi with special emphasis on *Penicillium* spp. in healthy conifer stands and infected with *H. annosum s.l.* and impact of forest age and soil type. The fifth (V) investigation was research of soil microorganism number, abundance and diversity in the stands of European aspen (*Populus tremula* L.) and hybrid aspen (*Populus tremuloides* Michx. × *P. tremul*a L.). The sixth (VI) investigation was about impact of six-year-long organic agriculture on soil microorganisms and crop disease suppressiveness in comparison to conventional agriculture.

2. Materials and methods

2.1. Sampling plots

Sampling plots of the investigations I, II, III and IV are listed in the Table 1. In the investigation V soil in four hybrid aspen and four European aspen stands growing on former agricultural soils was analyzed. They were located in the Forest research station "Kalsnava" (Kalsnava municipality), Forest research station "Auce" (Ukri municipality), and private aspen stands in Iecava and Ropazi municipality. In the investigation VI three fields of organic agriculture and four fields of conventional agriculture managed by State Priekuli Plant Breeding Institute, Latvia, were examined. In all analyzed fields there was sod-podzolic soil (Luvisols).

Table 1. Sampling plots and information about soil types and vegetation. Forest type according to the classification of Buss (1997)

Sampling plot, location	Soil type – Latvian soil classification (Karklins 2008)	Soil type – international classification (FAO WRB 2006)	Vegetation
Investigation I			
Forest1, Cesis district, Taurene	Typic podzol	Haplic Cambisols	*Myrtillosa* forest type[1] – spruce (*Picea abies*), pine (*Pinus sylvestris*), birch (*Betula pendula*), gray alder (*Alnus incana*)
Forest2, Valka district, Strenci	Typic podzol	Haplic Cambisols	*Vacciniosa* forest type – spruce (*Picea abies*), pine (*Pinus sylvestris*), birch (*Betula pendula*)
Forest3, Valka district, Strenci	Typic podzol	Haplic Arenosols	*Cladinoso-callunosa* forest type - pine (*Pinus sylvestris*) monoculture
Former1, Cesis district, Taurene	Eroded soil	Haplic Luvisols	Natural afforestation with spruce (*Picea abies*)
Former2, Cesis district, Taurene	Colluvial soil	Haplic Luvisols	Natural afforestation with spruce (*Picea abies*)
Meadow1, Jelgava district, Svete	Gleyic sod – podzolic soil	Stagnic Cambisols	Regularly cut and pastured
Meadow2, Jelgava district, Svete	Granular alluvial soil	Fluvic Cambisols	Regularly cut and pastured
Meadow2, Jelgava district, Svete	Pseudogley sod – podzolic soil	Endogleyic Umbrisols	Regularly cut and pastured
Field1, Jelgava district, Svete	Pseudogley sod – podzolic soil	Endogleyic Umbrisols	Arable land
Field2, Jelgava district, Svete	Sod – pseudogley soil	Mollic Luvic Stagnosols	Arable land
Field3, Jelgava district, Svete	Sod – gleyic soil	Endogleyic Umbrisols	Arable land

Table 1. *(continued)*

Sampling plot, location	Soil type – Latvian soil classification (Karklins 2008)	Soil type – international classification (FAO WRB 2006)	Vegetation
Investigation II			
Soil1, Malpils municipality	Sod-podzolic soil	Cutanic, Stagnic Albeluvisols	Forest type *Oxalidosa.* 40 years old spruce (*Picea abies*) stand.
Soil2, Malpils municipality	Illuvial humus podzol	Placic, Rustic, Albic, Folic, Stagnic Podzols	Forest type *Myrtilloso-polytrichosa.* 40 years old spruce (*Picea abies*) stand.
Investigation III			
Aa, Malpils municipality	Sod-podzolic soil	Cutanic Stagnic Albeluvisols	Forest type *Oxalidosa.* 40 years old spruce (*Picea abies*) stand.
Ab, Malpils municipality	Illuvial humus podzol	Placic Rustic Albic Folic Stagnic Podzols	Forest type *Myrtilloso-polytrichosa.* 40 years old spruce (*Picea abies*) stand.
Ba, Cesis district, Taurene	Typic podzol	Haplic Cambisols	*Myrtillosa* forest type[1] – spruce (*Picea abies*), pine (*Pinus sylvestris*), birch (*Betula pendula*), gray alder (*Alnus incana*)
Bb, Valka district, Strenči	Typic podzol	Haplic Cambisols	*Vacciniosa* forest type – spruce (*Picea abies*), pine (*Pinus sylvestris*), birch (*Betula pendula*)
Bc, Valka district, Strenči	Typic podzol	Haplic Arenosols	*Cladinoso-callunosa* forest type - pine (*Pinus sylvestris*) monoculture
Ca, Cb, Cc, Jekabpils district, Vipe	Drained peatland	Histosols	Pine (*Pinus sylvestris*), birch (*Betula pendula*), aspen (*Populus tremula*), gray alder (*Alnus incana*)
Investigation IV			
Pine 80, Ropazi municipality	Typic podzol	Folic Arenosols	Forest type *Myrtillosa.* 80 years old pine (*Pinus sylvestris*) stand.
Spruce 37, Ropazi municipality	Illuvial humic podzol	Folic Albic Podzols	Forest type *Oxalidosa.* 37 years old spruce (*Picea abies*) stand.
Spruce 47, Ropazi municipality	Stagnogley sod-podzolic soil	Folic Gleyic Calcic Luvic Planosols	Forest type *Oxalidosa.* 47 years old spruce (*Picea abies*) stand.
Spruce 28, Ropazi municipality	Illuvial humic podzol	Podzols	Forest type *Hylocomiosa.* 28 years old spruce (*Picea abies*) stand.
Spruce 62, Ropazi municipality	Typic podzol	Folic Albic Podzols	Forest type *Hylocomiosa.* 62 years old spruce (*Picea abies*) stand.
Spruce 141, Ropazi municipality	Typic podzol	Folic Podzol	Forest type *Hylocomiosa.* 141 years old spruce (*Picea abies*) stand.
Spruce 160, Ropazi municipality	Sod-podzolic soil	Folic Arenosols	Forest type *Vacciniosa.* 160 years old spruce (*Picea abies*) stand.
Spruce 50, Ropazi municipality	Humi-podzolic gley soil[b]	Rustic Folic Endogleyic Podzols	Forest type *Oxalidosa.* 50 years old spruce (*Picea abies*) stand.
Spruce 56, Ropazi municipality	Humi-podzolic gleyic soil[b]	Cutanic Albic Gleyic Luvisols	Forest type *Hylocomiosa.* 56 years old spruce (*Picea abies*) stand.

2.2. Soil sampling

In the investigation I soil samples were taken in three depths: 0-10 cm, 10-30 cm and 30-40 cm. In the investigations II, III, IV and V soil was analyzed in two depths within 0-30 cm deep upper soil layer. In the investigation VI soil was analyzed only in one depth, 10–15 cm. Samples were placed in sterile plastic bags (*Nasco* WHIRL-PAK) and stored at 4 °C for a few days until plating of cultivable microorganisms and after that stored at –20 °C.

2.3. Physical and chemical analysis of soil

Soil moisture content was determined according to the standard method ISO 11465. The pH of the soil samples was measured in distilled water according to the standard method ISO 10390. Soil physical and chemical analyzes were ordered to the laboratory of the Forest regeneration and establishment group of the Latvian State Forest Research Institute „Silava" (IV, V) or to the Soil laboratory of the Department of Botany and Ecology, Faculty of Biology, University of Latvia (II). The following methods were used: LVS ISO 11464 (2006) Soil quality – Pretreatment of samples for physico-chemical analysis, LVS ISO 11465 (2006) Soil quality – Determination of dry matter and water content on a mass basis – Gravimetric method, LVS ISO 11277 (2010) Soil quality – Determination of particle size distribution in mineral soil material – Method by sieving and sedimentation, LVS ISO 11261 (2002) Soil quality – Determination of total nitrogen - Modified Kjeldahl method, LVS ISO 10694 (2006) Soil quality – Determination of organic and total carbon after dry combustion (elementary analysis), LVS ISO 10693:1995 Soil quality – Determination of carbonate content – Volumetric method, LVS ISO 11466:1995 Soil quality – Extraction of trace elements soluble in aqua regia, LVS 398 (2002) Soil quality – Determination of total phosphorus. The content of humic acids was determined according to the method of Zaccone et al. (2009).

2.4. Analysis of culturable soil microorganisms

In order to estimate the number of colony forming units (CFU) of culturable filamentous fungi (CFF), yeasts and bacteria by a plate count method, soil sample dilutions were prepared by adding 10 g of soil to 90 ml of sterile distilled water. Suspensions were homogenized 1 h on a horizontal shaker. After that serial dilutions were prepared, and 0.1 ml of respective dilutions (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were analyzed. Agarised tryptic soy (Biolife, Italy) medium (TSA) or nutrient broth ("Int. Diagnostics Group", Great Britain) was used for the enumeration of saprophytic bacterial CFU in the investigation V and VI respectively. Incubation time was 3 days, the temperature 20±2 °C. Agarised malt extract (MEA) 30 g l^{-1}, pH 5.5 (Biolife, Italy) (I, II, III – VI) was used as a growing medium that is favorable for fungi, yeasts and maltose using bacteria. Rose Bengal agar (RBA) with chloramphenicol (Biolife, Italy) was used for enumeration of fungi and yeasts (V). Incubation time for fungi was 5 days, the

temperature 20±2 °C. CFU were expressed per gram of dry soil.

Genera of CFF were determined after 10 days of incubation according to morphological characteristics and light microscopy results. For estimation of the Shannon-Weaver diversity index (H') for the genera of CFF (H'_{CFF}) the following equation was used: $H'_{CFF} = -\Sigma\, p_j \log_2 p_j$, where p_j – relative abundance of particular genera of CFF.

2.5. Extraction of total soil DNA and quality control

Total soil DNA was extracted using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., USA) that uses harsh lysis for the DNA extraction. Samples (250 mg) were homogenized using horizontal Mixer Mill Type MM 301 (Retsch, Germany) at a maximal speed of 30 Hz (1800 oscillations per minute) for 10 minutes. The amount and purity of the DNA was determined spectrophotometrically using Ultrospec 3100 Pro (Amersham Biosciences, UK) at wavelength 230, 260 and 280 nm in order to assess the contamination with proteins and humic compounds (Yeates et al. 1998).

2.6. ARDRA of soil DNA

region that contains two internal transcribed spacers (ITS) and the 5.8S rDNA gene (ITS1-5.8S-ITS2) was amplified with the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). In order to obtain the fingerprints of bacterial populations, the 16S region of bacterial rDNA was amplified with primers FORB and REVB (Yeates et al. 1998; Edwards et al. 1989) (only in V).

The PCR reactions in Mastercycler Personal (Eppendorf, Germany) were carried out in 50 µl volume. The mixture contained 0.4 µl Hot Start Taq DNA Polymerase (5 u μl^{-1}), 5 µl 10X Hot Start PCR Buffer, 5 µl dNTP Mix, 2 mM each, 4 µl 25 mM $MgCl_2$, 0.75 µl Bovine Serum Albumin 20 mg ml^{-1} (all reagents from Fermentas, Lithuania), 1 µl of each 25 µM primer (OPERON Biotechnologies, Germany), 30.85 µl sterile distilled water and 1 µl of DNA template. The PCR conditions were as follows: the initial denaturation step of 4 min at 95 °C, 40 s of denaturation at 95 °C, 40 s of annealing at 52 °C, 1 min of primer extension at 72 °C (30 cycles) and final extension 10 min at 72 °C.

For ARDRA analysis with restriction endonucleases *Bsu*RI (Chabrerie et al. 2003) or *Eco*RI, the amplification products after the PCR were precipitated by 450 µl of 90% ethanol and 0.3 M sodium acetate (pH 5.0). The precipitated DNA was washed with 70% ethanol, air dried, dissolved in ddH_2O and digested with *Bsu*RI or *Eco*RI (Fermentas, Lithuania). Restriction fragments were visualized in 2% agarose gel or native 6% polyacrylamide gel electrophoresis using the Mighty Small™ II (Hoefer, USA) unit. Gels were stained with ethidium bromide, photographed and analyzed with software KODAK1D. For the calculation of the Shannon-Weaver diversity index (H') for the ARDRA results the following equation was used: $H'_{ARDRA} = -\Sigma\, p_j \log_2 p_j$, where p_j = relative intensity of individual band (Gabor et al. 2003).

2.7. Sequencing of ribosomal DNA

Sequencing of fungal ribosomal DNA for identification purposes was performed in the investigations II - VI. In total, 414 isolates representing dominant filamentous fungi were isolated from the plates used for the enumeration of CFF, subcultured on MEA and sequenced.

Genomic DNA from approximately 0.25 g of mycelia was extracted using the method developed by Cenis (1992) or PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., USA). Extracted DNA was amplified by PCR with primers ITS4 and ITS1F. After PCR 5 µl of amplified products were subjected to a sequencing protocol with BigDye Terminator Cycle Sequencing Kit (version 3.1, Applied Biosystems, USA). DNA fragments from fungal isolates from aspen stands in Kalsnava and Ukri (investigation V) and investigation IV were sequenced in CBS KNAW Fungal biodiversity centre, Utrecht, the Netherlands in the frame of the project EMbaRC. The sequencing of the samples from Iecava and Ropazi (investigation V) and in the investigations II, III, VI was performed at the Latvian Biomedical Research and Study Center. In the investigations IV, partly V and VI, the sequencing was done with both primers (ITS4 and ITS1F). In the investigation II and partly in V was done only with primer ITS1F. Double stranded sequences of PCR amplicons were assembled and single stranded sequences were processed using Staden Package 1.6.0. Homology search was done against the National Centre for Biotechnology Information GenBank nucleotide database using the Basic Local Alignment Search Tool or against data base Q-bank (www.q-bank.eu).

Taxonomic data based on sequenced ribosomal DNA regions of the isolates was used to calculate Sørensen's community similarity index C_s using the following equation: $C_s = 2C / A + B$, in which A and B are the number of species in samples A and B, respectively, and C is the number of species shared by the two samples (Izzo et al. 2006).

2.8. Quantitative PCR

In the investigations I, II, III and VI the total soil DNA extracts were analyzed with qPCR. In qPCR either the ITS1-5.8S-ITS2 rDNA region of *Ascomycota, Basidiomycota* and *Zygomycota* fungi with primers ITS1F and ITS4 or of *Trichoderma* spp. with the primers uTr and uTf (Hagn et al. 2007) was used to determine the amount of total fungal DNA and *Trichoderma* spp. DNA within the total soil DNA. Each soil sample was analyzed in three replicates.

The reactions in SmartCycler (Cepheid) or 7300 Real time PCR system (Applied Biosystems) were carried out in 25 µl containing 12.5 µl SYBR® Premix Ex Taq (TaKaRa) or Maxima™ SYBR Green qPCR Master Mix 2x (Fermentas), 1 µM of each primer and 1 µl of the DNA template. The PCR conditions: 30 s at 95 °C, (30 s, 95 °C; 30 s, 55 °C for primers ITS1F and ITS4 or 60 °C for primers uTr and uTf; 60 s, 72 °C) x 35 cycles for primers ITS1F and ITS4 or x 40 cycles for primers uTr and uTf.

2.9. The antagonism assay of *Penicillium* spp. and *H. annosum s.l.*

Fifty two *Penicillium* spp. isolates from agricultural soil, former agricultural soil (aspen stands) and forest soil including eight isolates from the investigation IV and two isolates from the spruce stands infected with *H. parviporum* from the investigation III were used in antagonism tests on 3% MEA against two isolates of *H. annosum s.s.* (strain number in Microbial Strain Collection of Latvia MSCL 1020, MSCL 1021), one isolate from the sampling plot S56 *H. annosum s.l.* and two isolates of *H. parviporum* (MSCL 980, MSCL 981). Incubation temperature was 20±2 °C. Each pairing with root rot isolates was done twice. Petri dishes were checked and colonies measured three times or more until complete overgrowth of the dish in approximately 30 days. The efficiency of *Penicillium* in suppressing radial growth was calculated using growth measurements after 7–9 days of incubation as follows: (C – T) / C × 100, where C is radial growth measurement of the pathogen in the control and T is radial growth of the pathogen in the presence of *Penicillium* (Asran-Amal et al. 2005).

2.10. Statistical analysis

The F-test, t-test ($\alpha = 0.05$) and correlation analysis were performed with Excel (Microsoft, USA). The correlation was characterised as weak if value of the correlation coefficient r was < 0.5, as medium weak if its value was < 0.8 and strong if its value was > 0.8 (Arhipova and Bāliņa 2000). Stepwise multiple regression analysis and cluster analysis of ARDRA fingerprints was done with the R package (R Development Core Team 2009).

3. Results

3.1. The influence of the land use upon abundance of soil fungi and species diversity of their communities: comparison of conventional and molecular methods of analysis

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Environmental and Experimental Biology (2011) 9: 9–21 *Original Paper*

The influence of the land use on abundance and diversity of soil fungi: comparison of conventional and molecular methods of analysis

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Abstract

This study examined fungal communities in 11 soil profiles. The objective was to apply conventional and molecular microbiology methods to obtain baseline information on the general characteristics of soil microbial communities in relation to land use – forest, former agricultural land, meadow and arable land. The number of filamentous fungi species and the total number of cultivable microorganisms showed a tendency to decrease with increasing depth. The diversity of fungi obtained with amplified rDNA gene restriction analysis was similar for all studied land use groups, diversity decreased with soil depth, especially in soils of former agricultural land and meadow. The proportion of fungal DNA as part of the total soil DNA was significantly higher in forest and abandoned land soil than in agricultural soil. The amount of *Trichoderma* spp. DNA was similar for all land groups, but its relative amount as percentage of total fungal DNA was higher in meadow and arable land soil. The land use type had a significant impact only on the diversity of cultivable soil fungi and fungal DNA amount. Soil depth and moisture content had a greater effects.

Key words: ARDRA, fungal diversity, Shannon-Weaver diversity index, soil, quantitative PCR, *Trichoderma*.
Abbreviations: AME, agarised malt extract; ARDRA, amplified ribosomal DNA gene restriction analysis; CFF, cultivable filamentous fungi; qPCR, quantitative PCR; CFU, colony forming units.

Introduction

Soil is a heterogeneous environment and different components of the solid fractions in soil (sand, silt, clay, organic matter etc.) provide variable microhabitats. Major interrelated factors affecting microbial diversity in soil include soil forming processes, physicochemical properties of soil, soil particle size distribution, vegetation and land use type (management). The relative effects of these factors differ in different soil types, horizons and climatic zones (Garbeva at al. 2004). Several studies investigating the impact of different plant cultures and soil types have shown, that the soil type has the greatest influence on the structure of microbial populations (Chiarini et al. 1998; Grayston et al. 1998; Buyer et al. 1999; Marschner et al. 2001; Wieland et al. 2001), although under some conditions the influence of the vegetation may prevail, e.g. the maize rhizosphere accommodates specific bacterial communities, the structures of which change with the developmental state of the plant (Baudoin et al. 2002). Many publications emphasize the influence of the land use (agricultural land, grassland, tropical forest and pasture, etc.) on the structure of soil microbial communities (Nusslein, Tiedje 1999; McCaig et al. 2001; Webster et al. 2002; Clegg et al. 2003; Drijber et al. 2003).

Abiotic conditions of soil (pH, moisture content and temperature) affect soil microorganisms in all soil land use types (Jurgensen et al. 1997; Hackl et al. 2004; Setälä, Mc Lean 2004; Borken et al. 2006; Ruisi et al. 2007; Zachow et al. 2009). It is known that fungi in soil constitute a significant part of the soil biomass and they have several important functions in soil, such as decomposition of organic material, nutrient cycling, formation of soil aggregates and mycorrhizal symbiosis (Kabir et al. 2003). Most (more than 80 %) soil fungi and bacterial species are not cultivable under laboratory conditions (Leckie 2005).

Two main approaches can be employed to study soil microbial communities – conventional plating of cultivable microorganisms and molecular methods that are independent of cultivation. Both approaches are associated

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with specific advantages and disadvantages. Only a small portion of all microorganisms in soil can be cultivated in standard laboratory conditions, and the isolation of pure cultures and lengthy effort are needed for the determination of taxonomic identity of the isolates. In contrast, molecular methods provide general insight into the genetic heterogeneity of soil microbial communities and allow to identify specific microorganisms without isolation, but they are highly dependent on the success of the isolation of DNA from soil, eventual presence of the DNA amplification or restriction inhibitors, choice of the primers, discriminating power of analysis etc. (Kowalchuk et al. 2006).

Amplified ribosomal DNA gene restriction analysis (ARDRA) can be used for genetic fingerprinting of simple commu-nities, populations or phylogenetic groups. High resolution discrimination can be obtained at the species level. In soil microbiology this method is used to determine diversity within phylogenetic or functional groups of microorganisms (Lynch et al. 2004). Digested PCR products can be run in polyacrylamide (Perez-de-Mora 2006) or in agarose gel (Smit et al. 1997; Schwieger, Tebbe 2000; Chabrerie et al. 2003). Usually at least two restriction enzymes are used and the obtained fragments can be analyzed as separate (Schwieger, Tebbe 2000; Klamer, Hedlund 2004) or combined (Wang et al. 2008) data sets.

The abundance of the specific groups of microorganisms in soil can be determined by quantitative PCR (qPCR) (Filion et al. 2003; Kabir et al. 2003; Kolb et al. 2003; Smits et al. 2004; Fierer et al. 2005). This method has been employed to quantify amounts of the genus *Trichoderma* fungi in soil by estimating their potential to counteract the activity of fungal plant pathogens (Cordier et al. 2006).

The objective of our investigation was to apply conventional and molecular microbiology methods in order to obtain baseline information about general characteristics of Northern temperate zone soil microbial communities depending on land use – forest, abandoned (former) agricultural land, meadow and arable land. We characterized and compared soil microbial communities employing conventional plating and molecular methods, ARDRA and qPCR. The number of cultivable microorganisms (colony forming units, CFU) was determined using the plate count method. Abundance of representatives of the typical fungal genera was determined by light microscopy of colonies. A robust culture-independent ARDRA and qPCR with universal fungal primers and *Trichoderma* spp. specific primers were used as molecular methods. The Shannon-Weaver diversity index (*H'*) was determined for the communities.

Materials and methods

Sampling plots, soil sampling and estimation of soil pH

Samples from soil profiles or outcrops were taken with an auger from the Ap, A1 and B horizons of agricultural soils and O, Ah and B horizons of forest soils (0 – 10 cm, 10 – 30 cm and 30 – 40 cm deep), on August 2007 in Jelgava district (Latvia), and on October 2007 in the Cesis and Valka districts (Latvia). The sampling sites were divided in the four groups: forest land; former agricultural land; agricultural land – meadow; agricultural land – conventional arable land. The characteristics of the soil profiles are given in Table 1. Samples were placed in sterile plastic bags (*Nasco* WHIRL-PAK) and stored at 4 °C for a few days until plating of cultivable microorganisms and later stored at –20 °C. The pH of the soil samples was measured in distilled water (ISO 10390). The moisture content of the soil was determined according to ISO 11465.

Extraction and quality control of total soil DNA

Total soil DNA was extracted from the collected samples by harsh lysis using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc.). Samples (250 mg) were homogenized using a horizontal Mixer Mill Type MM 301 (Retsch) at 1800 oscillations min^{-1} for 10 min. DNA from each sample was extracted twice. The amount and purity of the DNA was assessed by spectrophotometry (Ultrospec 3100 Pro; Amersham Biosciences) and by 1% agarose gel electrophoresis.

PCR and ARDRA of soil DNA

The fungal ribosomal RNA gene region containing two internal transcribed spacers and the 5.8S rRNA gene (ITS1-5.8S-ITS2) was amplified with primers ITS1F and ITS4 (Gardes, Bruns 1993). These primers amplify the ITS1-5.8S-ITS2 region of *Ascomycota*, *Basidiomycota* and *Zygomycota* fungi. Soil samples were analyzed undiluted, or in presence of some inhibitors in 1:10 and 1:100 dilutions.

In a Eppendorf Personal Mastercycler the reactions were carried out in 50 µL with 2 u of Hot Start *Taq* DNA Polymerase in 1× Hot Start PCR Buffer containing 0.2 mM each NTP, 8 mM $MgCl_2$ (Fermentas), 0.5 µM each primer (OPERON Biotechnologies) and 1 µl of DNA template. The PCR conditions were: 4 min at 95 °C, (40 s, 95°C; 40 s, 52 °C, 60 s, 72 °C) × 30 cycles and 10 min at 72 °C.

The PCR amplification products were precipitated by 450 µL of 96% ethanol and 3 M sodium acetate, pH 5.0 (19:1). After 15 min incubation at –20 °C, samples were centrifuged in a Sigma 1-15P centrifuge for 15 min at 14 000 rpm in room temperature, washed with 70% ethanol, dried and dissolved in 16 µL of sterile distilled water. The DNA was divided in two parts and digested with restriction endonucleases *Bsu*RI (Chabrerie et al. 2003) and *Eco*RI (Fermentas) separately. Restriction products were run in 2% agarose gels, photographed with a BioSpectrum AC Imaging System and analyzed with software KODAK1D. For the estimation of the Shannon-Weaver diversity index (H'_{ARDRA}) the following equation was used:

$H'_{ARDRA} = -\Sigma\, p_j \log_2 p_j$, where p_j is a relative intensity of individual band (Gabor et al. 2003).

Table 1. Soil profiles and their characterization. [1]The average pH (± SD) calculated from the pH measurements of all analyzed depths. [2]Sampling date 26.10.2007, average air temperature 4.02 °C. [3]Forest type according to the classification of Buss (1997). [4]Sampling date 27.10.2007, average air temperature 5.23 °C. [5]Sampling date 07.08.2007, average air temperature 20.61 °C. [6]Sampling date 08.08.2007, average air temperature 22.74 °C

Location	Soil profile	Soil type		Vegetation	Number of soil samples	pH	Soil moisture content at each depth (%)
Forest lands							
Cesis district, Taurene[2]	Forest1	Typical podzol	Haplic Cambisols	*Myrtillosa* forest type – spruce (*Picea abies)*, pine (*Pinus sylvestris*), birch (*Betula pendula*), gray alder (*Alnus incana*)	3	5.12 ± 0.23	42.5; 8.4; 8.0
Valka district, Strenči[4]	Forest2	Typical podzol	Haplic Cambisols	*Vacciniosa* forest type – spruce (*Picea abies*), pine (*Pinus sylvestris*), birch (*Betula pendula*)	3	5.30 ± 0.53	6.6; 3.0; 8.2
Valka district, Strenči[4]	Forest3	Typical podzol	Haplic Arenosols	*Cladinoso-callunosa* forest type – pine (*Pinus sylvestris*) monoculture	3	4.46 ± 0.62	4.5; 2.9; 45.0
Former agricultural lands							
Cesis district, Taurene[2]	Former1	Eroded soil	Haplic Luvisols	Natural afforestation with spruce (*Picea abies*)	3	7.53 ± 0.20	15.7; 19.3; 21.4
Cesis district, Taurene[2]	Former2	Colluvial soil	Haplic Luvisols	Natural afforestation with spruce (*Picea abies*)	3	6.68 ± 0.29	18.1; 17.6; 22.6
Agricultural lands, meadows							
Jelgava district, Svete[5]	Meadow1	Gleyic sod-podzolic soil	Stagnic Cambisols	Regularly cut and pastured	3	7.90 ± 0.31	10.2; 10.6; 8.3
Jelgava district, Svete[5]	Meadow2	Granular alluvial soil	Fluvic Cambisols	Regularly cut and pastured	3	7.44 ± 0.26	14.1; 13.2; 12.9
Jelgava district, Svete[5]	Meadow3	Pseudogley sod-podzolic soil	Endogleyic Umbrisols	Regularly cut and pastured	2	7.72 ± 0.06	10.7; 10.4
Agricultural lands, conventional arable lands							
Jelgava district, Svete[6]	Field1	Pseudogley sod-podzolic soil	Endogleyic Umbrisols	Arable land	2	7.55 ± 0.07	10.4; 9.8
Jelgava district, Svete[6]	Field2	Sod-pseudogley soil	Mollic Luvic Stagnosols	Arable land	3	7.69 ± 0.11	14.2; 12.7; 10.9
Jelgava district, Svete[6]	Field3	Sod-gleyic soil	Endogleyic Umbrisols	Arable land	2	6.96 ± 0.06	10.6; 13.2

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Analysis of cultivable microorganisms

In addition to the utilized molecular methods, cultivable microorganisms in three replicates were obtained from soil samples from forest and former agricultural land to estimate the CFU of filamentous fungi, bacteria and yeasts. Soil sample serial dilutions were prepared (Pepper et al. 1995; Alef, Nannipieri 1998). Agarised malt extract (AME) (30 g L^{-1}, pH 5.4 ± 0.2, Biolife) was used as a growth medium, as it supports growth of filamentous fungi, yeasts and some groups of bacteria. In our work, the total number of cultivable microorganisms was estimated as the number of CFU of the microorganisms per gram of dry soil on AME after 120 h at 20 ± 2 °C (Vanderzant, Splittstoesser 1992).

Genera of cultivable filamentous fungi (CFF) were identified using keys (Barnett 1957; Kiffer, Morelet 2000) and light microscopy of the morphology of pure cultures.

For the estimation of the Shannon-Weaver diversity index of CFF (H'_{CFF}) the following equation was used:

$H'_{CFF} = -\Sigma\, p_j \log_2 p_j$, where p_j is a relative abundance of particular genera of CFF.

Quantitative PCR

In addition to the methods described in previous chapters, total soil DNA from the upper horizons was subjected to qPCR. In qPCR either the ITS1-5.8S-ITS2 rDNA region of higher fungi with primers ITS1F and ITS4 or of *Trichoderma* spp. with the primers uTr and uTf (Hagn et al. 2007) was used to determine the amount of total fungal DNA and *Trichoderma* spp. DNA as part of the total soil DNA. Each soil sample was analyzed in three replicates.

In a SmartCycler (Cepheid) the reactions were carried out in 25 µL containing 12.5 µL SYBR® Premix Ex Taq (TaKaRa), 1 µM of each primer and 1 µL of the DNA template. The PCR conditions were: 30 s at 95 °C, (30 s, 95 °C; 30 s, 55 °C for primers ITS1F and ITS4 or 60 °C for primers uTr and uTf; 60 s, 72 °C) × 35 cycles for primers ITS1F and ITS4 or × 40 cycles for primers uTr and uTf. To quantify fungal DNA, qPCR using serial dilutions of DNA from pure cultures of *Heterobasidion parviporum*, *Penicillium lanoso-viride* MSCL 1 and *Trichoderma harzianum* MSCL 309 were performed and standard graphs were built (Fig. 1A). The average values of all three cultures were used for the calculation of the calibration curve for the total amount of the fungal DNA in soil. A standard graph (Fig. 1B) using serial dilutions of DNA of *T. harzianum* was used for the calculation of the concentration of trichodermal DNA in soil. In order to estimate the total copy number of the fungal DNA we assumed that the average fungal genome size was 35 Mb (Gregory et al. 2007) and that the average trichodermal genome size was 36.5 Mb (Kullman et al. 2005).

Statistical analysis

In order to characterize the influence of human activity upon soil microbial communities we classified the soil types in four land use groups according to the increasing intensity of human impact: 1, forest land; 2, former agricultural land; 3, meadow; 4, agricultural land. The F-test, t-test ($\alpha = 0.05$) and correlation analysis were conducted using MS *Excel*, and the program R was used for multiple regression analysis.

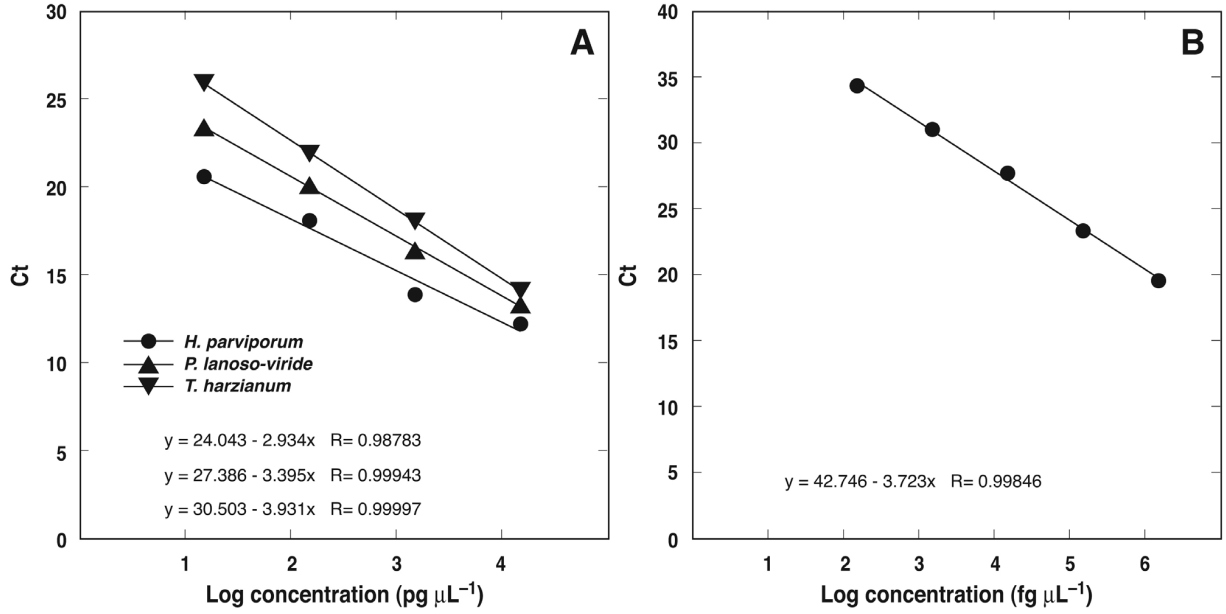

Fig. 1. Standard graphs for qPCR with *H. parviporum, P. lanoso-viride* and *T. harzianum* DNA dilution series with universal fungal primers (A) and standard graph with *Trichoderma harzianum* DNA dilution series with primers uTr and uTf (B).

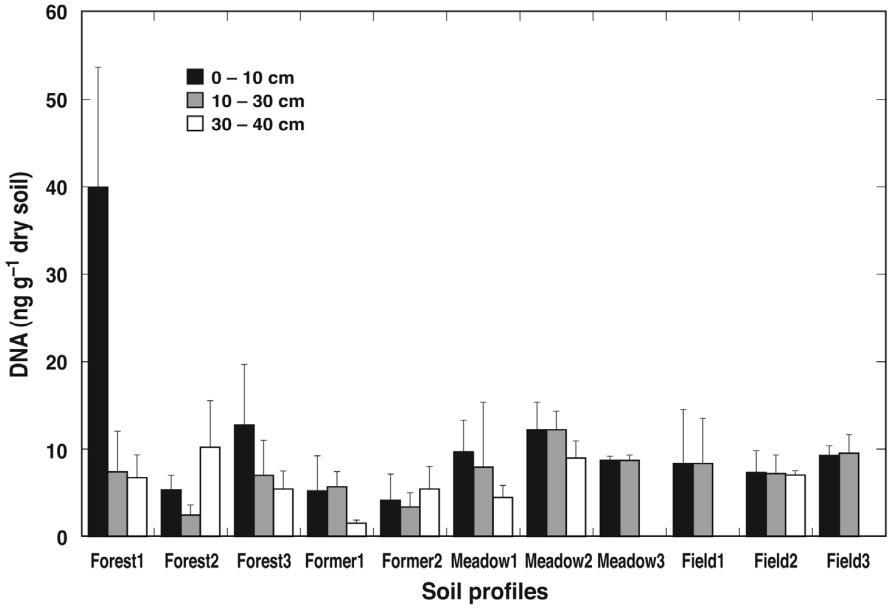

Fig. 2. Total soil DNA amount (mean ± SD, n = 2). Total soil DNA was extracted from 0.25 g of soil using PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc.).

Results

The amount of total soil DNA

The amount of total soil DNA ranged from 1.53 in deeper soil layers of former agricultural lands up to 30.28 μg g^{-1} of dry soil in upper horizons of forest soils (Fig. 2). The A_{260}/A_{280} ratio ranged from 1.6 to 1.8. In agarose gel electrophoresis the isolated DNA migrated as a diffuse band of a high molecular weight (>11 kb) region (data not shown).

Results of ARDRA

Typical ARDRA gels are shown in Fig. 3. We combined H'_{ARDRA} results from both restriction reactions (Fig. 4) as suggested by Wang et al. (2008).

Only some soil profiles (Forest1, Former2, Meadow1, Meadow3 and Field2) showed significant differences (t-test) in H'_{ARDRA} values between samples from different soil depths. In all soil profiles, the highest diversity of fungi was observed in the upper soil layer, with the exception of profiles Forest2 and Field2. In those two profiles the highest H'_{ARDRA} was observed at a depth of 10 – 30 cm. In profile Forest3 the fungal diversity was significantly higher ($p = 0.003$) than in the other two forest profiles.

In all the land use types, the mean fungal diversity H'_{ARDRA} was similar foe any of the examined sampling depths. In the 0 – 10 cm depth the H'_{ARDRA} varied from 2.61 ± 0.56 till 3.04 ± 0.24. At depth 10 – 30 cm, a significantly ($p = 0.002$) lower diversity occurred in meadows compared to other land use types – 2.26 ± 0.08 vs. 2.73 ± 0.29. In the deepest layer the H'_{ARDRA} varied from 1.95 ± 0.57 to 2.48 ± 0.48.

The number of cultivable microorganisms, dominant fungal genera and diversity of CFF

The number of CFF in soil samples (Fig. 5) from forest and former agricultural soils decreased significantly ($p < 0.05$) with increasing depth of sampling seen by comparing the upper soil layer (0 – 10 cm) with deeper layers (10 – 30 cm and 30 – 40 cm). The total number of CFU of

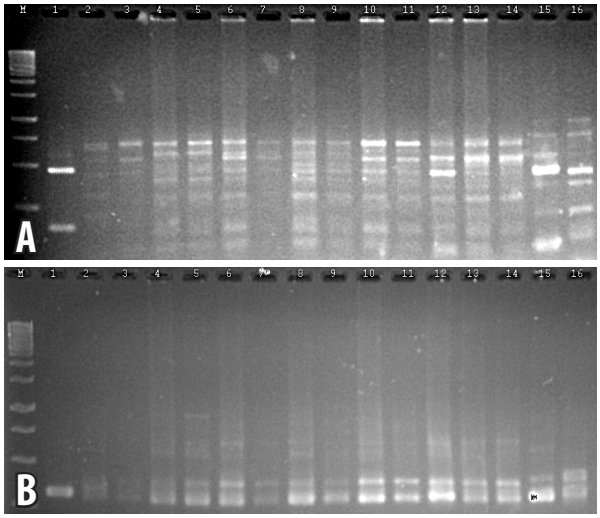

Fig. 3. Examples of ARDRA results in 2 % agarose gels. A – restriction with *Bsu*RI. B – restriction with *Eco*RI. Lanes in gels A and B: M-Gene Ruler 1 kb DNA Ladder (Fermentas). 1. Positive control with *T. harzianum* DNA. 2. Forest1, 30-40 cm. 3. Forest2, 0-10 cm. 4. Meadow2, 0-10 cm. 5. Meadow2, 10-30 cm. 6. Meadow3, 0-10 cm. 7. Meadow3, 10-30 cm. 8. Field1, 0-10 cm. 9. Field1, 10-30 cm. 10. Field2, 0-10 cm. 11. Field2, 10-30 cm. 12., 13. Field2, 30-40 cm. 14. Field2, 0-10 cm. 15., 16. Field2, 30-40 cm.

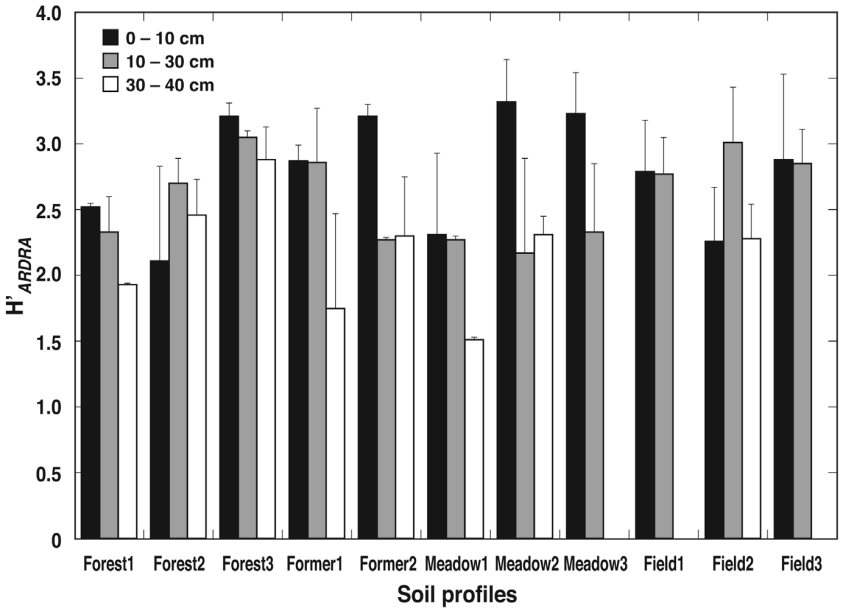

Fig. 4. Average Shannon-Weaver diversity index H'_{ARDRA} of fungal diversity in all analyzed land use groups (mean ± SD, n = 2).

microorganisms also decreased with increasing depth of sampling in soil profiles Forest1, Forest3 and Former1 (Fig. 6), but not in profiles Forest2 and Former2. In profile Forest1 the differences were statistically significant ($p < 0.05$) for all of the soil levels. In contrast to the other soils, profile Forest2 had a significantly ($p < 0.05$) increased number of cultivable microorganisms at a depth of 30 – 40 cm in comparison to the depth 0 – 30 cm. In soil profile Former2 the total number of cultivable microorganisms was similar in all horizons.

In general the amount of CFF was higher (statistically not significantly) in forest land soil in comparison with former agricultural lands, but the total count of CFU of microorganisms was higher (not significantly) in former agricultural land soil (Fig. 5, Fig. 6).

The most abundant genera and groups of fungi in soils are listed in Table 2. The identified fungi belonged either to *Ascomycota* or *Zygomycota*. Sterile mycelia and also *Penicillium* and *Mucor* were in all samples. Sterile mycelia were abundant in all of the analyzed profiles. In deeper soil layers of Forest1, Forest2 and Former2 profiles, sterile mycelia made up more than 90% of CFF.

H'_{CFF} index tended to be higher (not significantly) in forest soils than in former agricultural soils (Fig. 7).

Results of quantitative PCR

The highest amount of fungal DNA was found in forest soils, and the lowest in arable lands (no significant difference). The proportion of fungal DNA of the total soil DNA was the highest in the forest lands and former agricultural lands and lowest in meadows and fields (differences between these two groups was significant, $p = 0.02$). The amount of *Trichoderma* spp. DNA was similar in all the land use types, but the proportion of *Trichoderma* spp. DNA was higher in meadows and arable lands, in comparison to the other land use types, $p = 0.0006$ (Table 3).

Results of statistical analysis

According to multiple regression analysis (Table 4), sampling depth significantly affected all analyzed parameters; the number of CFF, total number of microorganisms, H'_{CFF}, H'_{ARDRA} and total soil DNA amount significantly decreased with depth. The strongest correlation was observed for number of CFF (Pearson correlation coefficient $r = -0.73$ to -0.83) and H'_{CFF} ($r = -0.54$ to -0.75).

In multiple regression analysis, soil moisture content had a significant positive effect on number of CFF ($p = 0.01$; $r = 0.23$). Similarly, the total soil DNA amount was positively affected by soil moisture ($p = 0.003$; $r = 0.42$). The fungal DNA amount and *Trichoderma* spp. DNA amount were positively affected by soil moisture ($p < 0.001$; $r = 0.71$ and $p = 0.01$; $r = 0.69$, respectively).

The mean air temperature on the sampling day (see Table 1) was significantly correlated with number of CFF ($p = 0.006$, $r = 0.35$) and fungal DNA proportion of total soil DNA amount ($p = 0.03$, $r = -0.65$) but not with total soil DNA ($p = 0.02$; no correlation). A strong negative correlation was observed with fungal DNA amount ($r = -0.81$) and with *Trichoderma* spp. DNA percentage of fungal DNA amount ($r = 0.85$) but this was not confirmed in multiple regression analysis.

Land use intensity had a negative impact on H'_{CFF} ($p = 0.004$, $r = -0.61$), total soil DNA amount ($p = 0.02$; $r = 0.24$), fungal DNA amount ($p < 0.001$; $r = -0.85$), and a positive impact on the proportion of *Trichoderma* spp. DNA ($p < 0.001$, $r = 0.90$).

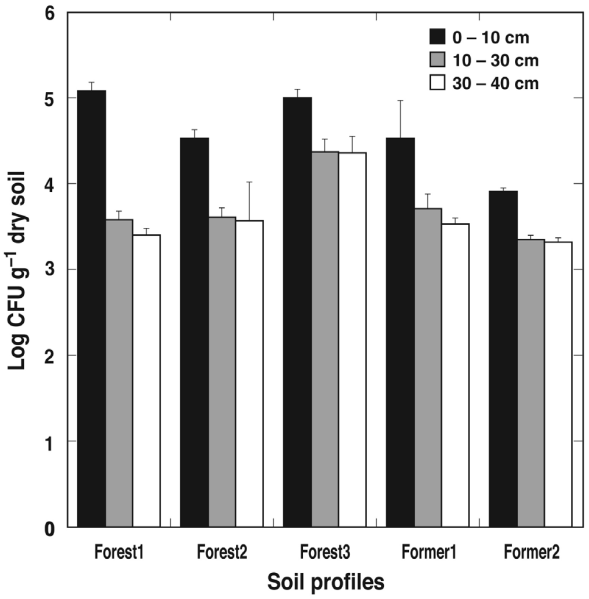

Fig. 5. The number of CFF in soil profiles in forests and former agricultural lands (mean ± SD, n = 3). The number of CFF was estimated on MEA after five days of incubation at 20 ± 2 °C.

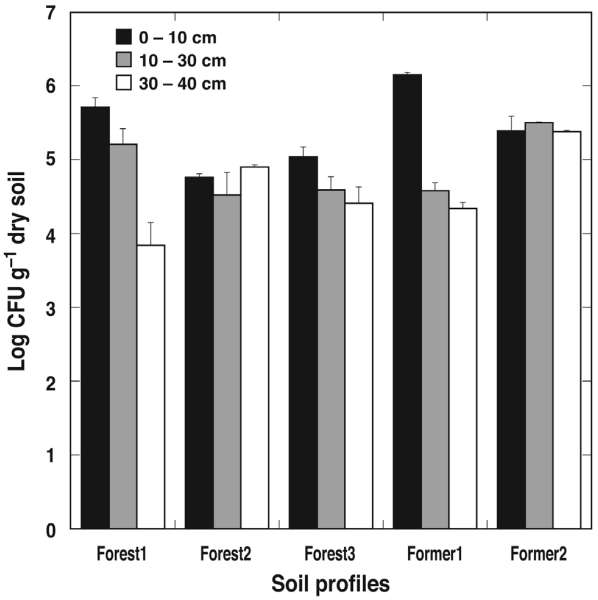

Fig. 6. Total number of cultivable microorganisms of soil profiles in forests and former agricultural lands (mean ± SD, n = 3). The number of cultivable microorganisms was estimated on MEA after five days of incubation at 20 ± 2 °C.

Table 2. Predominant fungal genera and groups

Fungi	Forest1	Forest2	Forest3	Former1	Former2
Ascomycota					
Penicillium	✓	✓	✓	✓	✓
Aspergillus	✓	✓	✓	✓	
Trichoderma	✓	✓	✓		
Verticillium			✓		
Acremonium					✓
Eladia saccula			✓		
Fusarium				✓	
Geomyces			✓		
Paecilomyces					✓
Spicaria					✓
Zygomycota					
Mucor	✓	✓	✓	✓	✓
Mortierella	✓				
Sterile mycelia	✓	✓	✓	✓	✓

Multiple regression analysis showed that soil pH affected significantly only the number of CFF (p = 0.01; r = –0.40), but correlation analysis showed a positive correlation also with H'_{CFF} (r = –0.53), and fungal DNA amount (r = –0.68), and negative correlation with proportion of affected fungal DNA (r = –0.57).

Discussion

Comparison of conventional and molecular methods

The correlation between the fungal diversity indexes

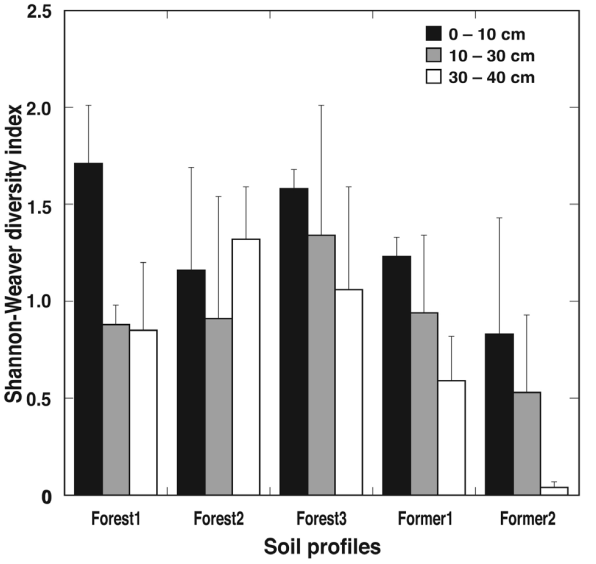

Fig. 7. Shannon-Weaver diversity index H'_{CFF} of soil profiles in forests and former agricultural lands (mean ± SD, n = 3). H'_{CFF} was calculated using CFU number of each CFF genus estimated on MEA after 10 days of incubation at 20 ± 2 °C.

obtained by conventional plating methods (H'_{CFF}) and by molecular methods (H'_{ARDRA}) was weak (r = 0.45). A stronger correlation of H'_{ARDRA} was found with number of CFF (r = 0.50 – 0.61), total soil DNA amount (r = 0.54 – 0.72), and fungal DNA amount (r = 0.58). Values of H'_{ARDRA} were always higher than those of H'_{CFF}. The diversity estimated by conventional methods used data only on filamentous fungi

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Table 3. The amount of fungal and *Trichoderma* spp. DNA in the upper soil layer (0 – 10 cm), n = 3

Profile	Total soil DNA (μg g^{-1} dry soil ± SD)	Fungal DNA [ng g^{-1} dry soil ± SD (% of total DNA)]	Number of rDNA copies g^{-1} dry soil	*Trichoderma* spp. DNA [ng g^{-1} dry soil ± SD (% of fungal DNA)]	Number of *Trichoderma* spp. DNA copies g^{-1} dry soil
Forest1	30.28 ± 13.78	1223.01 ± 247.06 (7.03)	$(3.24\pm0.65) \times 10^7$	10.27 ± 1.62 (0.79)	$(2.61 \pm 0.41) \times 10^5$
Forest2	4.17 ± 1.67	220.80 ± 22.22 (5.66)	$(5.84\pm0.59) \times 10^6$	1.57 ± 0.19 (0.64)	$(3.99 \pm 0.48) \times 10^4$
Forest3	9.98 ± 4.01	683.82 ± 116.44 (7.20)	$(1.81\pm0.31) \times 10^7$	2.33 ± 0.48 (1.01)	$(5.91 \pm 1.22) \times 10^4$
Average	14.81	709.21 (4.17)	1.88×10^7	4.72 (0.81)	1.20×10^5
Former1	8.09 ± 4.03	254.70 ± 39.15 (3.15)	$(6.74\pm1.04) \times 10^6$	2.14 ± 0.66 (0.84)	$(5.43 \pm 1.68) \times 10^4$
Former2	7.69 ± 5.00	260.84 ± 30.60 (3.39)	$(6.90\pm0.81) \times 10^6$	5.49 ± 0.33 (2.11)	$(1.39 \pm 0.08) \times 10^5$
Average	7.89	257.77 (3.27)	6.82×10^6	3.82 (1.48)	9.70×10^4
Meadow1	9.66 ± 3.61	136.00 ± 2.12 (1.41)	$(3.60\pm0.06) \times 10^6$	5.14 ± 0.26 (3.78)	$(1.30 \pm 0.07) \times 10^5$
Meadow2	12.18 ± 3.12	192.93 ± 7.46 (1.58)	$(5.11\pm0.20) \times 10^6$	5.55 ± 0.21 (2.87)	$(1.41 \pm 0.05) \times 10^5$
Meadow3	8.40 ± 6.18	106.68 ± 5.03 (1.27)	$(2.82\pm0.13) \times 10^6$	3.13 ± 0.63 (2.93)	$(7.94 \pm 1.60) \times 10^4$
Average	10.08	145.02 (1.42)	3.84×10^6	4.61 (3.19)	1.17×10^5
Field1	7.06 ± 0.47	103.44 ± 9.87 (1.47)	$(2.74\pm0.26) \times 10^6$	5.16 ± 0.40 (4.99)	$(1.31 \pm 0.10) \times 10^5$
Field2	5.27 ± 2.48	113.84 ± 23.83 (2.16)	$(3.01\pm0.63) \times 10^6$	3.05 ± 1.03 (2.68)	$(7.74 \pm 2.61) \times 10^4$
Field3	3.47 ± 4.28	84.47 ± 13.42 (2.43)	$(2.24\pm0.36) \times 10^6$	3.81 ± 0.16 (4.51)	$(9.67 \pm 0.41) \times 10^4$
Average	5.27	100.58 (2.02)	2.66×10^6	4.01 (4.06)	1.02×10^5

Table 4. Impact of different factors on the analyzed parameters. NS, not significant; NE, not estimated

	Number of CFF	Total number of microorganisms	H'_{CFF}	H'_{ARDRA}	Fungal DNA amount	*Trichoderma* spp. DNA amount
Sampling depth	p = 0.0001 (negative)	p = 0.01 (negative)	p = 0.007 (negative)	p = 0.005 (negative)	NE (negative)	NE (negative)
Soil moisture content	p = 0.01	NS	NS	NS	p < 0.001	p = 0.01
Soil pH	p=0.01 (negative)	NS	NS	NS	NS	NS
Average air temperature of the sampling day	p = 0.006	NS	NS	NS	NS	NS
Land use type	NS	NS	p = 0.004 (negative)	NS	p < 0.001 (negative)	NS
Multiple R^2	0.81	0.49	0.66	0.28	0.97	0.59
Probability of the model	p = 0.0003	p = 0.02	p = 0.001	p = 0.01	p < 0.001	p = 0.03

and excluded yeasts. In molecular analysis the diversity of yeast genomes also contributed to the estimated diversity, since they are amplified with primers ITS4 and ITS1F alongside with other representatives of *Ascomycota* or *Basidiomycota*. Further, conventional methods can identify cultivable microorganisms, while the H'_{ARDRA} index reveals the diversity "of some of the most abundant community members" (Kowalchuk et al. 2006). In other investigations the detection threshold of ARDRA gels is quite high, and can detect the DNA of two different strains in various proportions at ratios as low as 1/20 (Grundmann, Normand 2000).

In most soil profiles the highest H'_{ARDRA} and H'_{CFF} index was found in the upper soil layers. However, in two soil profiles (Forest2 and Field2), the highest diversity H'_{ARDRA} was found at a depth of 10 – 30 cm, and in the Forest2 soil profile the richest community of H'_{CFF} was in the deepest soil layer (30 – 40 cm). These differences were probably not caused by soil moisture or soil pH, but can be explained by the history of soil profile development (see further).

In profile Forest3, the estimated fungal diversity was significantly higher than in the other two forest profiles, which might be caused by different soil types (Haplic Arenosols in Forest3 compared to Haplic Cambisols in the

other two forest profiles) and/or with the associated forest vegetation type.

In North Carolina in two forest sites with loblolly pine (*Pinus taeda*) and mixed hardwood the highest fungal richness was in the O horizon (organic material and detritus), intermediate values were obtained in horizons L (litter) and A (organic matter-rich mineral soil), and the lowest in the B horizon (clay-rich mineral soil) (O'Brien et al. 2005). The authors found that in general the diversity and richness was correlated positively with clone library size.

The highest diversity of cultivable microfungi and yeasts in Austria was found in the top layer of the forest soil (0 – 15 cm) without temporal flooding (Wuczkowski et al. 2003). This layer of the soil profile was characterized as a zone of the highest degradation of the organic material. These results are similar to ours with the exception of the soil profile Forest2. In agricultural soils with conventional farming the highest diversity was recorded at a depth of 30 – 35 cm caused by agricultural practices (Wuczkowski et al. 2003). In our investigation the H'_{ARDRA} diversity in agricultural soil at a depth of 10 – 30 cm was similar or slightly higher than that in the upper soil layer. In the case of the profile Field2, the diversity was lower in deeper soil (depth of 30 – 40 cm). Since this soil layer was not analyzed in the other two fields, it is difficult to make any general conclusions about the fungal diversity in this depth.

In soils of Sweden, the fungal species richness investigated using terminal-restriction fragment length polymorphism was significantly higher in newly abandoned agricultural land than in actively used agricultural fields and forest (Klamer, Hedlund 2004). In our investigation former agricultural soils had the same diversity H'_{ARDRA} as other soil groups. The set-aside land in Sweden had not been used for five years and had not reached an equilibrium state with respect to competition between species, and thus represented higher diversity than soil of a forest, which represents a climax community. The abandoned agricultural lands studied in Latvia had not been used approximately for 15 years and thus the microbial community had progressed substantially toward a climax status.

The structure of dominant DNA fragments and consequently of the communities change at different soil depths, as shown in gel A (Fig. 3). This tendency was also observed regarding CFF; for example, in forest soils the amount of sterile mycelia proportionally increased with increasing depth. O'Brien et al. (2005) observed that mycorrhizal species predominate deeper in the soil profile whereas saprophytic species predominate in the litter layer. Further sequencing data will demonstrate if the sterile mycelia in our soil profiles was formed by mycorrhizal species.

The correlation between the number of CFU of *Trichoderma* spp. and *Trichoderma* spp. DNA amount was weak (r = 0.20). This can be explained by the fact that not all species or strains of *Trichoderma* genus can sporulate under standard laboratory conditions (Ellison et al. 1981; Schrüfer, Lysek 1990) and they may have been counted as sterile mycelia.

Distribution of cultivable microorganisms

The number of CFF and the total number of cultivable microorganisms tend to decrease with soil depth. An exception was soil profile Forest2 in which the highest number of cultivable microorganisms was at a depth of 30 – 40 cm, and soil profile Former2 in which the number of cultivable microorganisms was similar at all depths. This can be explained by the past history of profile development. In the profile Forest2 the upper 30 cm layer was composed of younger colluvial material with the original profile starting at 30 cm. The soil at Former2 was previously plaughed which destroyed the original stratification of the microorganisms.

A decrease in amounts of microorganisms in deeper soil horizons has been observed in investigations using different methods – phospholipid fatty acid analysis (Fierer et al. 2003; Fritze et al. 2000), ergosterol level (Krivtsov et al. 2007), and denaturing gradient gel electrophoresis (Krave et al. 2002). Nevertheless, some exceptions have also been described. For example, a bacterial peak has been observed at a 42.5-cm depth in the peat profile of a spruce (*Picea abies*) and birch (*Betula pubescens*) forest in Denmark (Ekelund et al. 2001) caused by partial anaerobic conditions, higher water content and higher organic matter content deeper in the soil. An even distribution of microorganisms without a decrease in their number with depth was found in a cryogenic weakly solidized loamy sandy pale soil of Yakutia (Ivanova et al. 2008). There is no permafrost in the subsoil of Latvia, but cryoturbation of the soil during winter may have some effect.

The CFF identified in our work are representatives of genera that have been isolated from a broad range of soils. For example, in the investigation of Wuczkowski et al. (2003), in forest soils of *Salix* and *Populus* stands and conventional agriculture soils the most abundant fungal genera were *Acremonium, Cladosporium, Penicillium, Cylindrocarpon* and *Trichoderma*. In boreal forest stands of *Picea mariana*, genera *Umbelopsis*, *Mortierella* and *Penicillium* were reported to be common (Summerbell 2005). In our work the most abundant genera were *Penicillium* and *Mucor* (in all analyzed soil profiles) as well as *Aspergillus* and *Trichoderma*, in some soil profiles also *Mortierella* and *Acremonium* were identified.

Quantification of fungal DNA

According to the qPCR results, fungal DNA contributed only 1.13 – 5.51% of the total extracted soil DNA (Table 3) which was several times lower amount than that found in North-Western France (Gangneux et al. 2011).

In forest lands and former agricultural lands with natural

afforestation the proportion of fungal DNA within the total soil DNA was significantly higher than in agricultural land types – 2.21 – 5.51% in forests and abandoned agricultural lands compared to 1.13 – 2.17% in currently used agricultural lands. Interestingly, the higher amount of total soil DNA of Forest1 (Fig. 1) in the upper soil horizon was not associated with a different population of fungal DNA compared to that in other forest soil samples. A strong correlation between fungal DNA amount and the period since last tillage was shown in an investigation carried out in North-Western France – the lowest fungal DNA amount was observed in fields with conventionally cultivated crops, and the highest in grasslands (Gangneux et al. 2011).

The amount of *Trichoderma* spp. DNA varied from 0.64 to 4.99 % of the total fungal DNA, and the highest relative abundance of *Trichoderma* spp. DNA was observed in agricultural soils.

Multiple linear regression analysis results (Table 4) showed that increased soil moisture content resulted in higher amounts of fungal DNA and *Trichoderma* spp. DNA. The amount of fungal DNA was affected also by land use type.

Higher fungal-bacterial DNA ratios have been previously observed in forest soil in comparison to pasture and cultivated soil (Lauber et al. 2008). In beech forest soil (Dystic Cambisol) in southern Germany the mean number of *Trichoderma* spp. ITS region copies was lower (1.2×10^6 g^{-1} of soil fresh mass) than in arable soil (Loamy Cambisol) – 1.3×10^6 g^{-1} of soil fresh mass (Hagn et al. 2003). Considering that the ITS region is amplified in fungal genome in even several 100 copies (Simon, Weiβ 2008), our calculation of *Trichoderma* genome copies (Table 3) was in the same range as the data obtained from soils in Germany.

Impact of different factors on the analyzed soil microorganisms

Multiple regression analysis showed that the number of CFF was negatively affected by increasing soil pH. In medium acid soils (forest soils – pH 4.46 – 5.30), fungal abundance was higher in comparison with neutral soils (all other soil groups – pH 6.68 – 7.90), since fungi prefer more acidic environments that bacteria. The impact of soil pH on soil microbial communities has been widely described previously. In boreal forests fungal biomass is very high in acid soil (pH < 4.1) (Högberg et al. 2007).

Soil moisture content in general has a positive impact on the number of CFF, fungal DNA amount and *Trichoderma* spp. DNA amount. Similarly, increasing moisture content was shown to cause higher biomass and *H'* index of soil fungi communities in Zoige Alpine Wetland (Feng et al. 2009).

Multiple regression showed that air temperature can have some effects on soil microbial populations. Study of the seasonality of microbial population dynamics has shown that soil microbial communities undergo a shift in function and genetic structure between winter and summer (Lipson et al. 2002).

Land use type affected the diversity of CFF, total soil DNA, fungal DNA amount and the proportion of *Trichoderma* spp. DNA. In a similar investigation in the southeastern United States in which hardwood forests, pine forests, cultivated and livestock pasture lands were compared, it was determined that the composition of fungal communities was most strongly correlated with specific soil properties (soil nutrient status) rather than land use types (Lauber et al. 2008).

We found no significant differences between fungal communities from meadows and arable lands – the diversity H'_{ARDRA} values were similar, while the proportion of fungal DNA and proportion of *Trichoderma* spp. DNA were slightly higher (not significant) in arable lands. Similarly, no substantial differences in *H'* indexes of arbuscular mycorrhizal fungi between meadows and arable lands in Central Europe were found by Oehl et al. (2003) but using other methods significant differences between soil groups were shown.

Conventional microbiological and molecular biology methods for investigating soil fungal communities showed similar results regarding relationships between depth and moisture and abundance of fungi. The numbers of CFF and the total number of cultivable microorganisms tended to decrease with increasing depth, which is in line with previous studies. There was a weak correlation between H'_{ARDRA} and H'_{CFF} diversity indexes, and the values were higher for H'_{ARDRA}. Both approaches showed higher fungal diversity at depths of 0 – 10 cm and 10 – 30 cm in comparison with a depth of 30 – 40 cm. The proportion of fungal DNA within the total soil DNA was significantly higher in forests and abandoned lands than in agricultural lands. The amount of *Trichoderma* spp. DNA was similar in all soil groups, but its proportion of the total fungal DNA amount was higher in meadows and arable lands.

An increase of land use intensity had a negative effect on the diversity of cultivable filamentous fungi (comparing forest lands with abandoned agricultural lands) and on the fungal DNA amount (comparing arable lands and meadows with forest lands and abandoned agricultural lands).

Our analysis showed that different soil types in Latvia show common features of distribution of fungal organisms, similar to the pattern found in other climatic zones and soil types. Natural phenomena (floods) and tillage causes changes in the distribution of soil fungal populations, which remain for many years. We demonstrated that molecular and conventional methods of analysis show rather weak correlation in determining total amount of soil fungi and their distribution among soil horizons. Both approaches showed the impact of soil moisture, land use type and air temperature on fungal communities. Correlation between fungal species diversity indices obtained by molecular and conventional plating methods was low. Molecular methods

gave higher diversity values, but more evenly distributed diversity of fungal species in different soil types and horizons.

The comparative analysis of fungal communities in soils under different types of natural forests, actively used and abandoned agricultural lands has provided baseline information about the fungal diversity and composition in these ecosystems although the obtained results are very variable due to the fact that each soil profile was unique in terms of soil type and land use.

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3.2. Monitoring seasonal changes of Northern Temperate zone spruce forest soil microbial populations

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Monitoring seasonal changes in microbial populations of spruce forest soil of the Northern Temperate Zone

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Abstract. Soil microbial populations in the Northern Temperate Zone have been poorly studied in comparison with extreme environments. The aim of the work was to study the seasonal changes in the microbial populations of spruce forest soil of the Northern Temperate Zone using classical methods of microbiology and molecular biology. Upper horizons in two *Picea abies* stands on sod-podzolic and illuvial humus podzol soil were analysed. Sampling was done monthly over a period of twelve months (May 2009–April 2010). Microbial communities in both experimental plots showed different responses to the analysed environmental factors. In the sod-podzolic soil only the fungal DNA amount was significantly higher in the rest period (October–April) in comparison with the active vegetation period (May–September) and the number of *Penicillium* spp. colonies was larger in the active vegetation period. In the other soil the number of maltose utilizing bacteria, yeasts, and *Penicillium* spp. and other culturable filamentous fungi was significantly higher in the active vegetation period, while the fungal DNA amount was elevated in the rest period. Although ARDRA did not reveal differences, sequencing of 84 fungal isolates showed different compositions of the communities. Sørensen's index between the plots was low (0.29). Comparing the active vegetation period with the rest period, the index was higher (0.48). Although all tested fungal isolates from the rest period were able to grow at 4 °C, none of them showed psychrotrophic growth characters.

Key words: forest soil, filamentous fungi, active vegetation period, rest period, Shannon–Weaver diversity index, qPCR.

INTRODUCTION

Several studies are available about the impact of seasonal changes upon soil microorganisms in arctic soils (Nemergut et al., 2005), deserts, tropical soils, and other soils in extreme environments (Dion, 2008). Comparatively few investigations have been published about microbial populations in the soils of the Northern Temperate Zone (NTZ) in which climatic conditions during winter are charac-

terized by permanent or temporal snow cover and a significant decrease of topsoil temperature below zero.

Functional diversity of bacterial populations was assessed in sandy soils over a climatic gradient in Western Canada using sole-carbon-source utilization analyses (Staddon et al., 1998). Response of soil bacteria and endomycorrhizal fungi to nitrogen deposition in northern forest ecosystems was investigated in north-western Lower Michigan (Waldrop et al., 2004). Studies on the seasonal dynamics of the microbial population of alpine dry meadow sites in the Colorado Rocky Mountains revealed that the microbial community undergoes a shift in function and genetic structure at the change of winter and summer seasons (Lipson et al., 2002). Microbial biomass and activity increase to maximal levels in late winter under the snow and then decline during snowmelt. This results in an increased availability of nitrogen, which is used by plants and the developing summer microbial community (Brooks et al., 1998; Lipson et al., 1999, 2000; Schmidt et al., 2004). Potential allelochemicals, such as simple phenolic compounds, are released from fresh litter in autumn and make up carbon and energy sources for the microbial community that develops in autumn and winter (Lipson et al., 2000, 2002). These potential allelochemicals are consumed before plant growth starts in spring (Schmidt & Lipson, 2004). In NTZ ecosystems litter decomposition (Zimov et al., 1993; Schmidt & Lipson, 2004) and gas accumulation (e.g., CO_2, CH_4, N_2O) take place under the winter snow packs. In the soil of jack pine (*Pinus banksiana*) stands in Western Canada Staddon et al. (1998) found significant positive correlations between the diversity of microbial populations and the soil pH, average daily temperature in July, mean annual temperature, degree-days above 18 °C, and degree-days above 5 °C.

The aim of our work was to analyse seasonal changes in the microbial populations in NTZ spruce (*Picea abies*) forest soil from two closely located forest stands with the same dominant tree species, but with different relief, soil, and vegetation types using classical methods of microbiology and molecular biology. The results confirmed that microbial populations are active under the snow during winter, while the two analysed soils showed different responses to environmental factors.

MATERIALS AND METHODS

Experimental plots

Two 40-years-old Norway spruce (*Picea abies* (L.) Karst.) stands in Malpils municipality (ca 30 km east of Riga, Latvia) were analysed. The plots were located ca 200 m from each other. Soil1 was sod-podzolic soil (Cutanic, Stagnic Albeluvisols) with *Oxalidosa* forest type. Soil2 was illuvial humus podzol (Placic, Rustic, Albic, Folic, Stagnic Podzols) with *Myrtilloso-polytrichosa* forest type (forest type classification according to Buss (1997), and soil types according to Latvian and FAO WRB (2006) soil classifications). Forest stands were heavily infected with the root rot fungus *Heterobasidion parviporum* (Grantina et al., 2010).

Soil1 was located at the upper part of a hill on moraine sediments and Soil2 in the depression of the relief on glaciofluvial sediments. The topography caused differences in the soil drainage between the experimental plots; the groundwater level was higher in Soil2. During winter only upper horizons of Soil1 were frozen (I–III). The snow cover of ca 0.50 m thickness lasted from January until mid-March.

Meteorological data

The raw meteorological data were obtained from the database of the Latvian Environment, Geology and Meteorology Centre, as recorded at the Meteorological Station in Riga. Average daily and monthly air temperature and total precipitation for every month were calculated.

Soil sampling and physico-chemical analysis

Soil samples for the microbiological analysis were taken in the middle of every month (9th–12th day, May 2009–April 2010) from 0–10 cm and 11–30 cm depths. Observations were divided into active vegetation period (AVP; May–September, average daily temperature $t > 10\,°C$) and rest period (RP; October–April, $t < 10\,°C$). A soil sampling plot was a circle with three transects on which nine samples (100 g) were taken at both depths at distances of 0.3 m, 1.0 m, and 3.0 m from the centre. Samples from the same depth were combined, resulting in three samples from both depths. Samples were placed in sterile plastic bags (*Nasco* WHIRL-PAK), stored at +4 °C for a few days until the plate count of culturable microorganisms was made, and after that stored at –20 °C. These samples were used also for the estimation of soil moisture content according to ISO 11465. For all other physico-chemical analyses soil samples (1–3 kg) were taken from each soil horizon in September 2009. Chemical analyses were made using (Expert Panel on Soil, 2006).

Analysis of culturable soil microorganisms

In order to estimate the number of colony forming units (CFU) of culturable filamentous fungi (CFF), yeasts, and maltose utilizing bacteria by a plate count method, soil sample dilutions were prepared by adding 10 g of soil to 90 mL of sterile distilled water. Suspensions were homogenized for 1 h on a horizontal shaker. After that serial dilutions were prepared, and 0.1 mL of dilutions 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were used. Agarised malt extract (MEA; 30 g/L, pH 5.5, from Biolife) was used as a growing medium that is favourable for fungi, yeasts, and certain groups of bacteria. Further in the text all these groups together are referred to as culturable microorganisms. The incubation time was 5 days and the temperature was 20±2 °C. The CFU were expressed per gram of dry soil. Every soil sample was analysed in three replicates.

Genera of filamentous fungi were determined after 10 days of incubation according to morphological characteristics and light microscopy results.

Extraction of total soil DNA and quality control

Total soil DNA was extracted with a PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc.), which uses harsh lysis for DNA extraction. Samples (250 mg) were homogenized using a horizontal Mixer Mill Type MM 301 (Retsch) at a maximal speed of 30 Hz (1800 oscillations/minute) for 10 min. The amount and purity of the DNA were established spectrophotometrically using Ultrospec 3100 Pro (Amersham Biosciences) at wavelengths of 230, 260, and 280 nm in order to determine the contamination with proteins and humic compounds (Yeates et al., 1998).

ARDRA of soil DNA

For the amplified ribosomal RNA gene analysis (ARDRA) the fungal rRNA gene region that contains two internal transcribed spacers (ITS) and the 5.8S rRNA gene (ITS1-5.8S-ITS2) was amplified with the primers ITS1F and ITS4 (Gardes & Bruns, 1993).

The reactions in Eppendorf Mastercycler Personal were carried out in 50 μL volume. The mixture contained 0.4 μL of Hot Start *Taq* DNA Polymerase; 5 μL of 10X Hot Start PCR Buffer; 5 μL of dNTP Mix, 2 mM each; 4 μL of 25 mM $MgCl_2$; 0.75 μL of Bovine Serum Albumin 20 mg/mL (all reagents from Fermentas); 1 μL of each 25 μM primer (OPERON Biotechnologies); 30.85 μL of sterile distilled water; and 1 μL of DNA template. The polymerase chain reaction (PCR) conditions were as follows: the initial denaturation step of 4 min at 95 °C, 40 s of denaturation at 95 °C, 40 s of annealing at 52 °C, 1 min of primer extension at 72 °C (30 cycles), and final extension 10 min at 72 °C.

For ARDRA analysis with restriction endonuclease *Bsu*RI (Chabrerie et al., 2003) the amplification products after the PCR were precipitated by 450 μL of 90% ethanol and 0.3 M sodium acetate (pH 5.0). The precipitated DNA was washed with 70% ethanol, air dried, dissolved in ddH2O, and digested with *Bsu*RI (Fermentas). The restriction products were visualized in 6% polyacrylamide gel electrophoresis using a Mighty Small™ II (Hoefer) unit. Gels were photographed and analysed with KODAK1D software. For the calculation of the Shannon–Weaver diversity index (H') the following equation was used: $H' = -\sum p_j \log_2 p_j$, where $p_j =$ relative intensity of individual band (Gabor et al., 2003).

Quantitative PCR

In quantitative PCR (qPCR) the ITS1-5.8S-ITS2 rRNA gene region of higher fungi with primers ITS1F and ITS4 was used to determine the amount of total fungal DNA within the total soil DNA. Combined soil DNA samples for each soil depth were analysed in three replicates for every month's samples.

As both forest stands were infected with *H. parviporum*, we monitored with special interest the DNA amount of antagonistic fungi *Trichoderma* spp. of this tree pathogen (Korhonen & Stenlid, 1998). In order to estimate the amount of

Trichoderma spp. DNA, qPCR with the primers uTr and uTf (Hagn et al., 2007) was performed. Samples of every second month were used, each analysed in three replicates.

The reactions were carried out in 25 µL volume. The mixture contained 12.5 µL of Maxima^{TM} SYBR Green qPCR Master Mix 2x (Fermentas), 1 µL of each 25 µM primer, 9.5 µL of sterile distilled water, and 1 µL of the DNA template. The PCR conditions on 7300 Real time PCR system (Applied Biosystems) were as follows: initial denaturation 60 s at 95 °C; 40× (30 s of denaturation at 95 °C, 30 s of annealing at 55 °C for primers ITS1F and ITS4 or 60 °C for primers uTr and uTf, 30 s of primer extension at 72 °C). Calibration curves were built using serial dilutions of DNA from pure cultures of *Phlebiopsis gigantea* MSCL 702 and *Trichoderma harzianum* MSCL 309. In more detail the method is described in (Grantina et al., 2011).

Sequencing

A total of 84 isolates (45 sporulating, 39 sterile mycelia, i.e. not sporulating when kept at +4 °C for several months) representing dominant filamentous fungi were isolated from the plates used for the enumeration of CFF and subcultured on MEA. Genomic DNA from approximately 0.25 g of mycelia was extracted using the method developed by Cenis (1992). The extracted DNA was amplified in PCR with the primers ITS4 and ITS1F. Afterwards 5 µL of amplified products was subjected to a sequencing protocol with BigDye Terminator v. 3.1 Cycle Sequencing Kit with primer ITS1F. The sequencing of the samples was performed in the Latvian Biomedical Research and Study Center. The obtained sequences were analysed using Staden Package 1.6.0. and checked against the database of the National Centre for Biotechnology Information using the Basic Local Alignment Search Tool for identification.

From the data of the sequenced isolates Sørensen's community similarity index C_s was calculated using the following equation: $C_s = 2C/A + B$, in which A and B are the number of species in samples A and B (Soil1 and Soil2), respectively, and C is the number of species shared by the two samples (Izzo et al., 2006).

Screening for psychrotrophic and mesophilic fungi

For growth temperature tests 29 strains (19 from RP, 10 from AVP) were used. Colonies were incubated at 4±2 °C and at 20±2 °C on MEA. Growth (expressed as colony diameter) was assessed several times during 20 days and growth rate (expressed as mm/day) was calculated.

Statistical analysis

The *F*-test, *t*-test ($\alpha = 0.05$), and correlation analysis were made with Excel (Microsoft, USA). Multiple regression analysis of the results and cluster analysis of ARDRA fingerprints were performed with the R package (R Development Core Team, 2009).

RESULTS AND DISCUSSION

Results of the meteorological data analysis

The average air temperature and total monthly precipitation during the analysed period are given in Fig. 1. The highest average air temperature at the meteorological station in Riga was recorded in June (+16.68 °C), the lowest in January (–10.41 °C). The smallest amount of precipitation was recorded in May (9.70 mm) and the highest in June (136.80 mm).

Soil characterization

Data on the element content and total nitrogen content in each horizon of both soil profiles are listed in Table 1. Information about soil texture is given in Table 2. Certain differences in the element content between the profiles are caused by soil formation processes and later influence of geological sediments and topography: moraine contains more exchange cations; nitrogen accumulates in the lower parts of the relief.

In either experimental plot there were no statistically significant differences of soil moisture between sampling depths (Fig. 2). However, due to the landscape

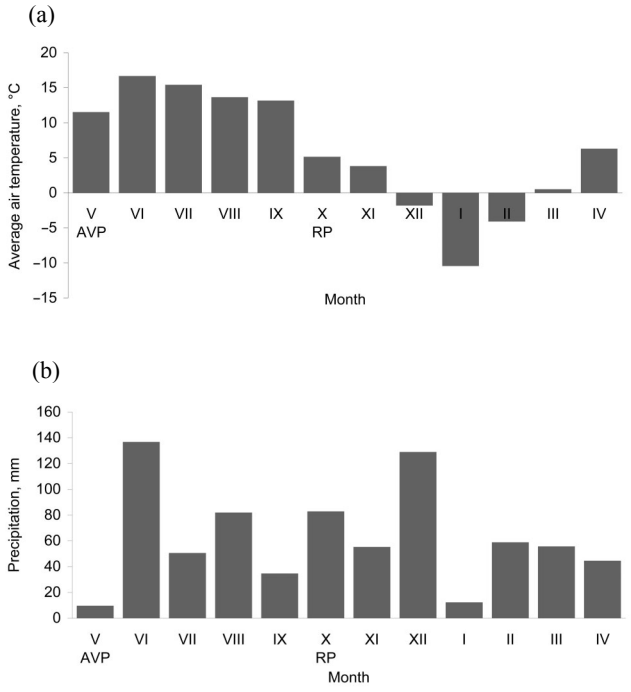

Fig. 1. Average air temperature (a) and total monthly precipitation (b) in the observation station at Riga during the analysed period. For the calculations raw meteorological data obtained from the database of the Latvian Environment, Geology and Meteorology Centre were used.

Table 1. Element content by soil horizons in the experimental plots

Horizon	Depth, cm	Ca, mg kg^{-1}	Mg, mg kg^{-1}	K, mg kg^{-1}	Na, mg kg^{-1}	P, mg kg^{-1}	N, %	pH_{KCl}
Soil1								
O	0–2	824.67±109.86	334.40±15.95	618.00±351.64	60.00±3.46	142.32±1.16	0.63±0.00	3.22±0.02
Ah	3–19	70.07±13.58	272.93±25.00	814.00±365.46	80.00±15.10	146.51±1.54	0.11±0.00	3.68±0.02
B	20–35	109.73±13.67	213.40±12.39	1140.67±49.57	70.00±10.00	103.13±1.34	0.06±0.00	3.98±0.01
EB	36–45	462.07±110.72	273.33±54.79	1318.00±3.46	98.00±10.58	63.76±0.77	0.06±0.00	3.56±0.01
Bt 1	46–81	2664.00±270.25	338.67±137.29	1320.00±3.46	140.67±51.47	76.69±0.59	0.00	3.95±0.00
Bt 2	82–130	2696.67±9.02	292.80±24.31	1282.00±10.39	125.33±23.44	125.80±1.32	0.00	4.62±0.02
Soil2								
O	0–11	2262.00±326.56	49.60±3.41	846.67±371.07	86.00±12.49	306.04±0.78	0.37±0.32	2.52±0.02
AhE	12–22	355.47±13.0	7.47±3.78	196.00±27.06	106.00±34.00	241.65±1.32	0.31±0.00	2.42±0.02
E	23–30	76.40±21.54	15.93±12.47	100.00±16.00	81.33±8.33	24.99±0.45	0.06±0.00	3.42±0.01
Bs 1	31–45	132.87±16.15	78.20±9.89	178.67±15.01	82.00±13.11	49.66±0.59	0.06±0.00	3.73±0.01
Bsg	46–61	223.53±53.53	247.07±33.62	182.00±7.21	71.33±11.02	72.09±0.97	0.06±0.00	3.88±0.01
Bs 2	62–102	310.60±4.85	230.80±17.17	200.00±19.29	76.00±16.37	82.96±0.77	0.00	4.04±0.02
R	103–135	409.47±100.82	252.73±30.66	249.33±36.90	54.00±3.46	111.29±1.38	0.00	4.08±0.01

Table 2. Soil granulometry by soil horizons in the experimental plots

Horizon	Clay, %	Silt, %	Sand, %	Characterization
Soil1				
O	Organic horizon			
Ah	10.9	24.7	64.4	Sandy loam
B	11.5	25.9	62.6	Sandy loam
EB	19.1	25.3	55.6	Sandy loam
Bt 1	22.5	26.7	50.8	Sandy clay loam
Bt 2	20.4	29.6	50	Loam
Soil2				
O	Organic horizon			
AhE	8.9	15.5	75.6	Loamy sand
E	3.8	5.2	91	Sand
Bs 1	6.2	2.8	90	Sand
Bsg	4.9	2.5	92.6	Sand
Bs 2	5.1	1.5	93.4	Sand
R	4.2	2.6	93.2	Sand

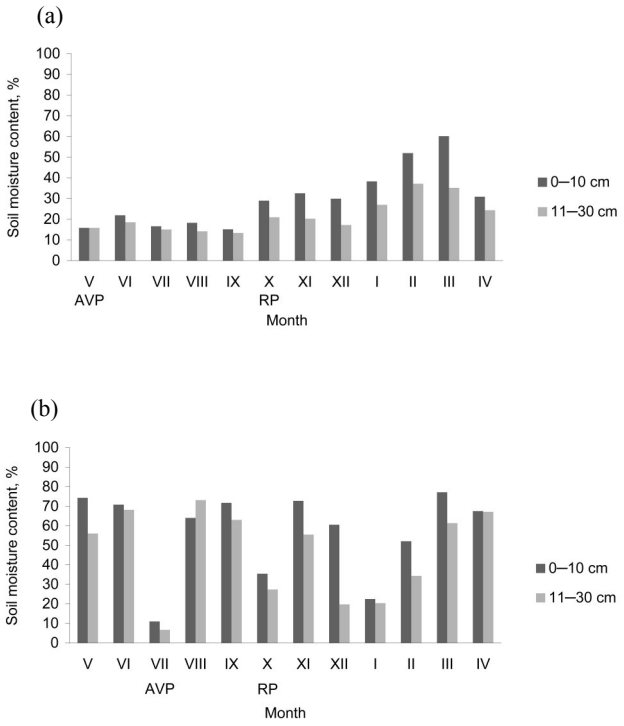

Fig. 2. Changes in soil moisture content during the analysed period in sampling plots Soil1 (a) and Soil2 (b) at two sampling depths (n = 1). Composite soil samples from each depth were used to estimate soil moisture content.

topography the moisture content was on average significantly higher in Soil2 ($F = 11.308$; $p < 0.001$). The topography of the plot and the presence of sphagnum moss caused ample fluctuations in its moisture content (6.69–72.55%). Still, in general there were no statistically significant differences of moisture content in Soil2 between the AVP and RP whereas in Soil1 the moisture content was significantly higher during the RP in comparison with the AVP ($F = 22.538$; $p < 0.001$).

The soil types and forest types of both analysed experimental plots are common in Latvia and in the NTZ. *Oxalidosa* and *Myrtilloso-polytrichosa* forests cover 19.5% and 3.6%, respectively, of the whole forest territory of Latvia. Sod-podzolic soil is the most common soil type in Latvia, but illuvial humus podzol is common only in lowlands (Karklins et al., 2009).

Soil chemical analysis showed that the soils of both experimental plots were very poor in nitrogen (0.63% in Soil1 and 0.37% in Soil2) in comparison with average values for the same forest types (1.8% in *Oxalidosa* forests and 1.5% in *Myrtilloso-polytrichosa* forests) in Latvia (Bardule et al., 2009).

The texture of Soil1 was typical of *Oxalidosa* forests, while Soil2 had a decreased content of clay and silt, but an increased content of sand in comparison with average soils of the *Myrtilloso-polytrichosa* forests in Latvia (Bardule et al., 2009).

Results of analysis of culturable microorganisms

Changes in the number of CFF in the experimental plots during the investigated period are presented in Fig. 3a, b. In Soil1 the numbers of obtained fungal colonies did not differ significantly between the AVP and RP and the colonies were equally distributed at both sampling depths (on average $(4.70 \pm 3.95) \times 10^4$ CFU g^{-1} dry soil). In Soil2 significant differences were observed between the two sampling depths ($F = 6.049$; $p < 0.05$) and between the AVP and RP ($F = 2.883$; $p < 0.05$; 2.15×10^5 and 1.84×10^4 CFU g^{-1} dry soil, respectively), accompanied by a remarkable variability within the 12 months; for example, low numbers of CFF were detected in July, October, January, and February.

The numbers of CFU of yeasts and maltose utilizing bacteria are shown in Fig. 3c, d. In Soil1 they were significantly different between the two sampling depths in the AVP ($F = 1.150$; $p = 0.020$; $(1.32 \pm 0.41) \times 10^6$ CFU g^{-1} dry soil at 0–10 cm depth, $(6.65 \pm 4.38) \times 10^5$ CFU g^{-1} dry soil at 11–30 cm depth), but similar at both depths in the RP. In general, differences in CFU numbers in Soil1 between the AVP and RP were not significant. In Soil2 the numbers of this group of soil microorganisms were not statistically significantly different between the two sampling depths but differed significantly ($F = 68.048$; $p = 0.032$) between the AVP and RP, being $(2.28 \pm 1.88) \times 10^6$ CFU g^{-1} dry soil on average and $(3.62 \pm 3.51) \times 10^5$ CFU g^{-1} dry soil, respectively.

So on average bacterial and fungal populations were in higher numbers in the AVP only in Soil2. Higher microscopic counts of bacteria in summer were recorded also by Lipson et al. (2002) in alpine dry meadow soils, but fungal

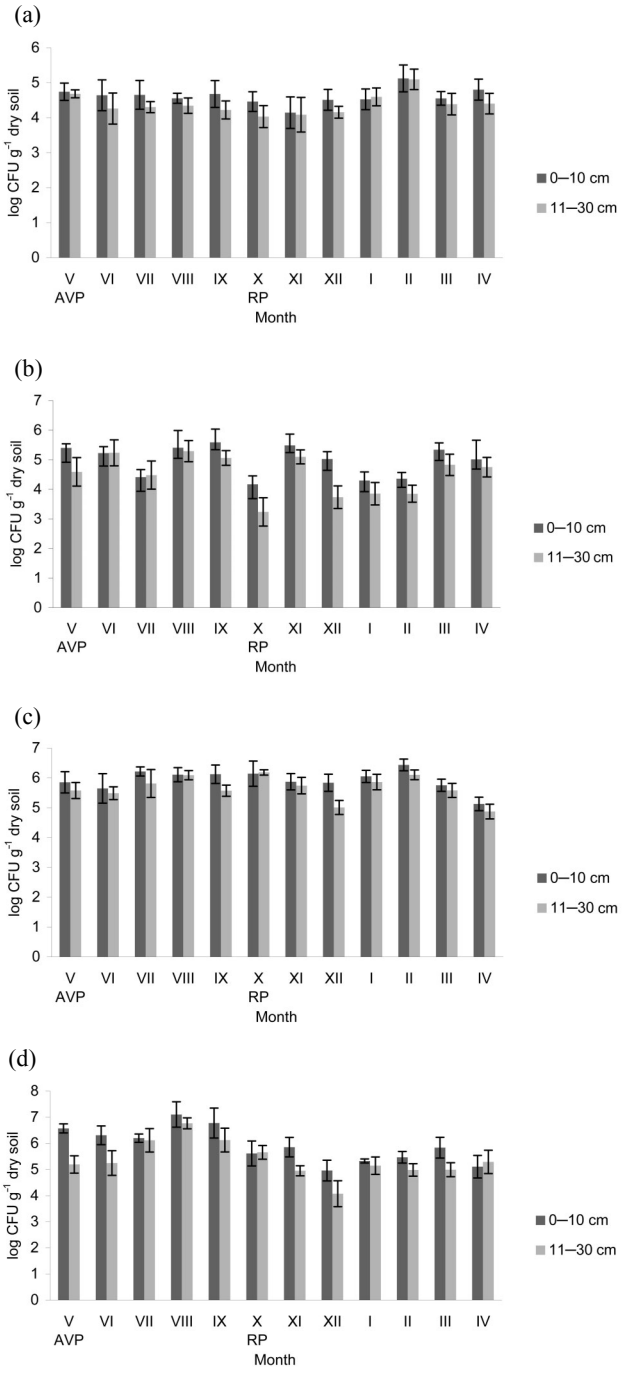

Fig. 3. Changes in the log number of CFF (a, b) and yeasts and maltose utilizing bacteria (c, d) during the investigated period in sampling plots Soil1 (a, c) and Soil2 (b, d) at two sampling depths (± SD; $n = 9$). Results were obtained after five days of incubation on MEA at the temperature of 20 ± 2 °C.

abundance expressed as length of hyphae (m g^{-1} soil) was increased in winter. These soils differ from our experimental plots in terms of vegetation and soil type, and they have at least two times higher nitrogen content in the A horizon (7.4 g N kg^{-1}). The authors concluded that winter microbial communities utilize more complex substrates such as cellulose but to a lesser extent simple compounds than do the summer communities.

As to particular dominant CFF genera on MEA, the percentage of *Penicillium* spp. was on average significantly higher during the AVP than during the RP in both plots: 33.20% versus 16.52% ($F = 2.407$; $p = 0.010$) in Soil1 and 38.36% versus 21.42% in Soil2 ($F = 2.415$; $p = 0.016$) (Fig. 4). Certain species such as *P. canescens* (one isolate) and *P. montanense* (four isolates) were detected only in the AVP, isolates of *P. spinulosum* (four isolates in total) were obtained in both periods according to sequencing results (Table 3).

Members of the genus *Mortierella* were evenly abundant in both the AVP and the RP in Soil1: 9.16% and 8.38%, respectively. In Soil2 the percentage of *Mortierella* spp. was low in the period July–January (on average 3.12%), but high in the periods May–June (29.93%) and February–April (24.71%). Looking at the particular species according to sequencing results, isolates of *M. alpina* (one isolate) and *M. gamsii* (one isolate) were detected only in the RP and *M. humilis* (one isolate) in the AVP, but isolates of *M. macrocystis* (four isolates in total) were detected in both periods.

The percentage of *Trichoderma* spp. was on average low in both plots, and at several sampling times *Trichoderma* spp. were not detected at all, e.g., in May–July and November–December in Soil1, and in October–November in Soil2. Detailed identification of fungal isolates using rRNA gene sequencing revealed that *Hypocrea viridescens* (March, one isolate) and *T. asperellum* (February, two isolates) were recovered only in the RP, but *H. pachybasioides* (six isolates in total) and *T. viride* (eight isolates in total) occurred both in the RP and AVP.

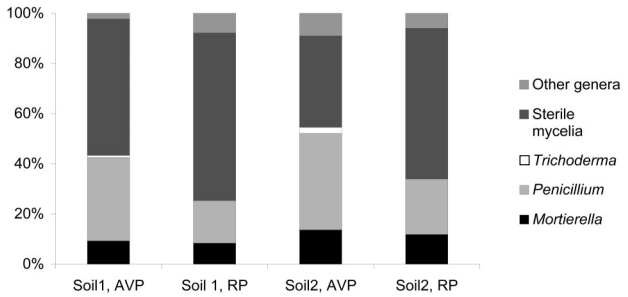

Fig. 4. Average percentage of the dominant CFF genera in the AVP and RP in the two sampling plots. Other genera include members from such genera as *Aspergillus*, *Botrytis*, *Cladosporium*, *Geomyces*, *Gilmaniella*, and *Mucor*. Results were obtained after ten days of incubation on malt extract agar at the temperature of 20 ± 2 °C.

Table 3. Fungal isolates of Soil1 and Soil2 identified by sequencing

Isolate[a]	CFU g^{-1} dry soil	Homolog sequence in NCBI	Max identity, %	Strain in MSCL[b]
Soil1, AVP				
Beauveria bassiana	10^5	GU566276.1	99	–
Beauveria geodes	10^4	U19037.1	98	968
Geomyces pannorum var. *asperulatus*	10^3	AJ938166.1	99	–
Hypocrea pachybasioides	10^3	FJ860796.1	100	–
Metarhizium anisopliae var. *anisopliae*	10^3	EU307900.1	99	950
Mortierella humilis	10^5	AJ878778.1	99	967
Mortierella macrocystis (1)	10^3	AJ878782.1	98	–
Mortierella macrocystis (2)	10^3	AJ878782.1	99	965
Mortierella sp. (1)	10^5	EU240040.1	99	–
Mortierella sp. (2)	10^4	EU240040.1	99	–
Penicillium canescens	10^5	DQ658168.1	100	–
Penicillium spinulosum	10^5	GU566247.1	99	–
Scytalidium lignicola	10^3	DQ093703.1	99	955
Trichocladium opacum	10^4	FN386299.1	100	958
Trichoderma viride	10^4	FJ481123.1	99	–
Umbelopsis ramanniana	10^3	DQ888724.1	99	–
Soil1, RP				
Beauveria bassiana	10^3	GU566276.1	99	960
Geomyces pannorum	10^3	FJ590611.1	99	947
Geomyces pannorum var. *asperulatus*	10^3	AJ938166.1	99	962
Geomyces vinaceus (1)	10^5	AJ608972.1	100	–
Geomyces vinaceus (2)	10^5	AJ608972.1	99	–
Hypocrea pachybasioides (1)	10^3	AY240841.1	99	–
Hypocrea pachybasioides (2)	10^4	AY240841.1	99	944
Hypocrea sp.	10^3	EU294196.1	99	–
Hypocrea viridescens	10^3	EU871022.1	99	–
Mortierella alpina	10^4	FJ161918.1	100	959
Mortierella gamsii	10^4	DQ093723.1	100	948
Mortierella macrocystis (1)	10^5	AJ878782.1	87	–
Mortierella macrocystis (2)	10^5	AJ878782.1	99	–
Mortierella sp. (1)	10^5	FJ810149.1	99	–
Mortierella sp. (2)	10^3	FJ810149.1	99	–
Mortierella sp. (3)	10^3	FJ810149.1	100	–
Mortierella sp. (4)	10^3	GQ302682.1	99	–
Paecilomyces carneus	10^5	AB258369.1	99	961
Penicillium sp.	10^5	FJ379821.1	99	–
Penicillium spinulosum	10^4	GU566247.1	99	–
Trichosporon porosum	10^5	AF414694.1	99	949
Umbelopsis ramanniana (1)	10^5	DQ888724.1	99	–
Umbelopsis ramanniana (2)	10^5	EU484205.1	97	941
Umbelopsis ramanniana (3)	10^4	DQ888724.1	98	–
Zygomycete	10^5	EF152531.1	90	–

Continued overleaf

Table 3. *Continued*

Isolate[a]	CFU g^{-1} dry soil	Homolog sequence in NCBI	Max identity, %	Strain in MSCL[b]
Soil2, AVP				
Beauveria geodes	10^3	U19037.1	99	–
Lecythophora mutabilis	10^4	HM036599.1	99	–
Mortierella sp.	10^4	FJ810149.1	99	–
Penicillium montanense (1)	10^5	AF527058.1	100	–
Penicillium montanense (2)	10^5	AF527058.1	100	–
Penicillium montanense (3)	10^4	AF527058.1	100	–
Penicillium montanense (4)	10^3	AF527058.1	100	942
Penicillium spinulosum	10^4	GU566247.1	99	–
Pezizomycete	10^5	GQ153212.1	82	–
Pochonia bulbillosa (1)	10^3	AB378551.1	99	–
Pochonia bulbillosa (2)	10^4	AB378551.1	99	964
Pseudeurotium bakeri	10^5	HM036612.1	100	–
Tolypocladium inflatum	10^5	GU354362.1	99	970
Trichoderma viride (1)	10^3	FJ872073.1	99	946
Trichoderma viride (2)	10^4	FJ481123.1	99	–
Umbelopsis isabellina	10^3	AJ876493.1	97	951
Soil2, RP				
Aspergillus cervinus	10^3	AY373845.1	98	963
Beauveria caledonica	10^3	AY532003.1	99	971
Geomyces pannorum var. *asperulatus*	10^3	AJ938166.1	99	–
Hypocrea pachybasioides (1)	10^4	AY240841.1	99	–
Hypocrea pachybasioides (2)	10^3	GU062213.1	98	–
Hypocrea pachybasioides (3)	10^3	AY240844.1	99	–
Lecythophora mutabilis	10^3	HM036599.1	99	956
Mortierella sp. (1)	10^4	EU240040.1	91	–
Mortierella sp. (2)	10^3	FJ810149.1	100	–
Mortierella sp. (3)	10^5	EU240039.1	99	–
Penicillium spinulosum	10^5	GU566247.1	99	952
Podospora appendiculata	10^3	AY999126.1	92	953
Pseudeurotium bakeri (1)	10^3	HM036612.1	99	–
Pseudeurotium bakeri (2)	10^3	HM036612.1	100	943
Rhizosphaera kalkhoffii	10^5	AY183366.1	100	954
Tolypocladium inflatum	10^4	GU354362.1	99	957
Trichoderma asperellum (1)	10^3	FJ605246.1	99	966
Trichoderma asperellum (2)	10^3	FJ605246.1	99	–
Trichoderma viride (1)	10^3	FJ481123.1	99	969
Trichoderma viride (2)	10^3	FJ481123.1	99	–
Trichoderma viride (3)	10^3	FJ481123.1	99	–
Trichoderma viride (4)	10^3	FJ481123.1	99	945
Trichoderma viride (5)	10^3	FJ481123.1	99	
Zygomycete (1)	10^5	AM292200.1	96	–
Zygomycete (2)	10^3	AM292200.1	96	–
Zygomycete (3)	10^3	AM292200.1	96	–
Zygomycete (4)	10^3	EF152547.1	92	–

[a] Number in the parentheses indicates different isolates.
[b] Microbial Strain Collection of Latvia.

Several sequenced *Trichoderma* isolates were obtained as sterile mycelia on agar plates and did not sporulate until the end of the laboratory experiments in May 2010. Seasonality studies of *Trichoderma* species in spruce forest soil in Canada showed that members of this genus are more influenced by the competition among soil fungi than environmental factors such as soil moisture content (Widden & Abitbol, 1980). The small proportion of *Trichoderma* spp. in the soil fungal community can explain why trees in both stands were heavily infected with *H. parviporum*.

Representatives of the genus *Mucor* were present in a low percentage: on average 1–2% in Soil1, with the exception of the upper soil layer in May (9.69%), November (21.23%), and April (11.38%) when a larger proportion of this group of moulds was found among the isolates. In Soil2 *Mucor* spp. were more abundant in May–November (on average 9.28%) in comparison with December–April (1.28%). *Gilmaniella* spp., *Geomyces* spp., and *Aspergillus cervinus* were isolated only in the RP. *Botrytis* was isolated only in the AVP. Several insect pathogens were isolated from the analysed soils: *Beauveria bassiana* (RP, AVP), *B. caledonica* (RP), *B. geodes* (AVP), and *Metarhizium anisopliae* (AVP).

Table 3 gives an overview of 84 sequenced fungal isolates (45 sporulating, 39 sterile mycelia). The sequence database of the National Center for Biotechnology Information (NCBI) and Blast algorithm were used for sequence comparison; in most cases the sequence similarity exceeded 98%. One member of every species identified was deposited in the Microbial Strain Collection of Latvia.

Sequencing revealed different composition of the communities in Soil1 and Soil2: only five species were common in both experimental plots (*B. geodes*, *G. pannorum*, *H. pachybasioides*, *P. spinulosum*, and *T. viride*). From Soil1 19 unique species were isolated and identified, but in the case of Soil2 their number was 16. The corresponding Sørensen's community similarity index C_s was 0.29. In Soil1 six species were common in the AVP and RP (*B. bassiana*, *G. pannorum*, *H. pachybasioides*, *M. macrocystis*, *P. spinulosum*, and *U. ramanniana*). The corresponding C_s was 0.48. In Soil2 five species were common in both periods (*L. mutabilis*, *P. spinulosum*, *P. bakeri*, *T. inflatum*, *T. viride*, and *U. isabellina*). The corresponding C_s was 0.48.

Results of the screening for psychrotrophic and mesophilic fungi

Several fungal species isolated from the soil samples of the RP have been detected in arctic environments or recorded as growing at low temperatures (such as 0 °C): *G. vinaceus*, *G. pannorum*, *M. alpina* (Bergero et al., 1999), and also *T. porosum* (Danielson & Davey, 1973). Although all our isolates of these fungi were able to grow at 4 °C, their growth was not impeded at 20 °C either.

Several species were typically isolated only in the AVP; for example, *P. bulbillosa*, *S. lignicola*, *M. anisopliae* var. *anisopliae*, and *P. montanense*. From these species *M. anisopliae* var. *anisopliae* was not able to grow at low temperatures.

In laboratory conditions (in vitro) all the tested isolates showed mesophilic growth, none of them was psychrotrophic. The members of *Trichoderma*/*Hypocrea* had the highest growth rates among all strains at 20 °C and at 4 °C: 5.94–13.17 mm/day and 1.43–1.98 mm/day, respectively. Also the members of the genus *Mortierella* had high growth rates: 5.68 and 2.17 mm/day; an exception was the isolate of *Mortierella alpina* from the RP with slower growth rates: 2.24 and 0.85 mm/day. One isolate of *T. inflatum* from the RP and one isolate of the same species from the AVP showed identical growth rates at 20 °C and 4 °C: 1.41 ± 0.21 and 0.30 ± 0.09 mm/day, respectively. From all 29 isolates tested only *M. anisopliae* var. *anisopliae* was not able to grow at 4 °C. The isolate of *R. kalkhoffii* from the RP showed identical growth rates (0.57 ± 0.16 and 0.52 ± 0.09 mm/day) at both temperatures up to the 10th day of incubation, but later it started to grow significantly faster at 20 °C (1.08 ± 0.12 and 0.47 ± 0.06 mm/day). The comparative growth rate of the isolated strains in laboratory experiments is indicative. It may not reflect the actual growth rate of the same strains in soil (in situ), although the correlation between in vitro and in situ data is expected to be positive; for example, the intensive growth rate of *Trichoderma*, which was found in our experiments, is the basis for the use of these fungi in plant protection in situ.

Total soil DNA amount and results of qPCR

The success of the molecular methods depends on the quantity and quality of the extracted total soil DNA. Our results of the total DNA amount of 10.03 ± 4.29 μg g^{-1} dry soil in Soil1 and 18.17 ± 9.59 μg g^{-1} dry soil in Soil2 are similar or higher than those found in other investigations where MoBio kits have been used: 11.1 ± 0.3 μg g^{-1} dry agricultural soil (Thakuria et al., 2008), 1.46 ± 3.7 μg g^{-1} dry podzolic soil (Carrigg et al., 2007); 1.06 ± 0.35 μg g^{-1} dry wetlands soil (Ning et al., 2009). Further molecular analysis of the DNA isolated from soil may be impeded by the presence of various inhibitors; many of them belong to humic compounds (Ning et al., 2009). We used Bovine Serum Albumin as a scavenger of potential inhibitors in the PCR reactions.

Changes in the amount of the total soil DNA in soil samples at the experimental plots are shown in Fig. 5a, b. The total soil DNA amount was similar at both sampling depths and in the RP and AVP, but it differed significantly ($F = 5.009$; $p < 0.01$) between the experimental plots: 10.03 ± 4.29 μg g^{-1} dry soil in Soil1 and 18.17 ± 9.59 μg g^{-1} dry soil in Soil2.

Changes in the amount of the fungal DNA during the analysed period are shown in Fig. 5c, d. No significant differences were observed at either analysed soil depth or in either experimental plot. However, in both experimental plots there were statistically significant differences between the AVP and RP. In Soil1 the fungal DNA amount was significantly ($F = 8.854$; $p = 0.013$) lower (8.08 ± 5.97 ng g^{-1} dry soil, excluding the value of the depth 10–30 cm in September) during the AVP than during the RP (112.40 ± 61.55 ng g^{-1} dry soil, excluding the

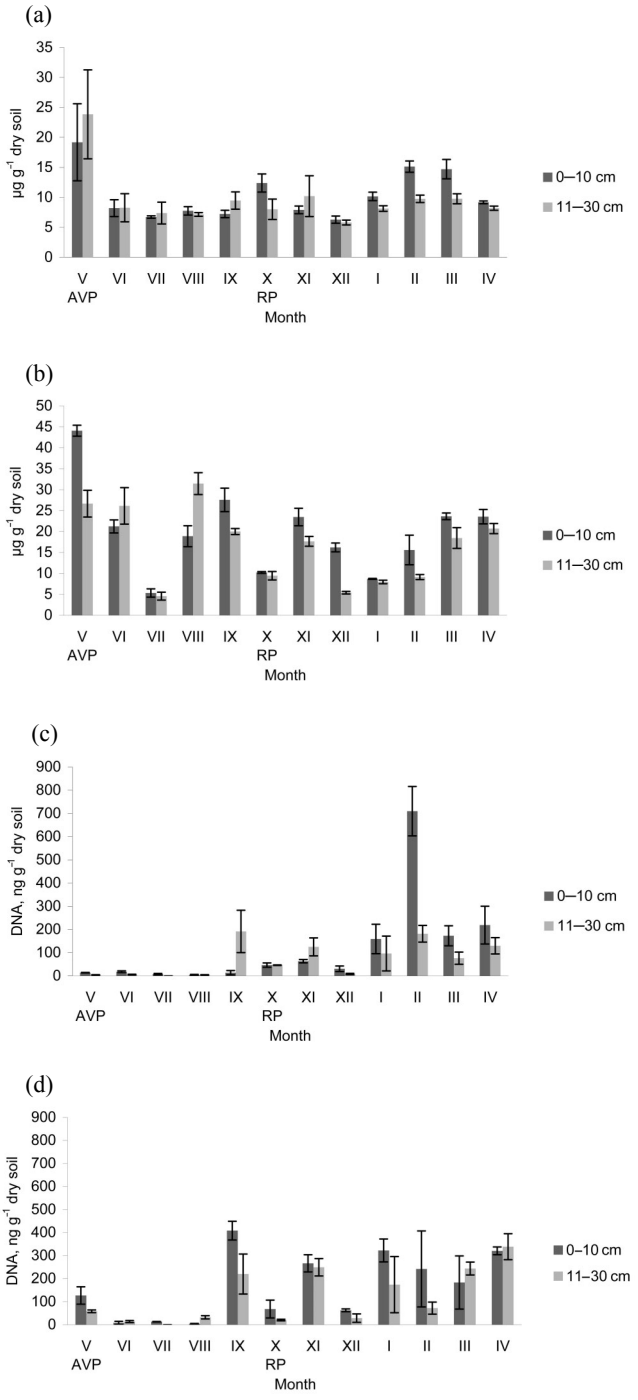

Fig. 5. Amount of total soil DNA (a, b) extracted with PowerSoil™ DNA Isolation Kit and total fungal DNA (c, d) estimated using qPCR in sampling plots Soil1 (a, c) and Soil2 (b, d) at two depths (±SD; $n = 3$) in the investigated period.

values of the upper soil layer in February and the lower soil layer in December). Similarly, the fungal DNA amount in Soil2 was significantly higher during the RP than during the AVP ($F = 1.331$; $p = 0.035$). During the RP the fungal DNA amount constituted on average 1.37% of the total soil DNA in Soil1 and 1.16% in Soil2. In the AVP the fungal DNA amount constituted a much lower proportion of the total soil DNA: on average 0.26% in Soil1 and 0.39% in Soil2. In both experimental plots the differences between the AVP and RP were statistically significant ($F = 3.125$; $p = 0.003$ and $F = 3.382$; $p = 0.003$).

The total amount of the fungal DNA increased at lower air temperatures in both experimental plots. This is in line with other investigations where it was determined that fungal populations dominate under the snow cover and that winter fungal populations are adapted to colder temperatures and more complex substrates (Lipson et al., 2002). It seems that cultivation independent methods such as fungal abundance in terms of hyphal length or molecular methods are more suitable for the studies of seasonal changes of fungal populations than the plate count method.

Our results agree with the observations that microbial biomass and activity increase to maximal levels in late winter under the snow and then decline during snowmelt due to the limitation of carbon sources (Brooks et al., 1998; Lipson et al., 1999, 2000; Schmidt et al., 2004). Our data demonstrated that during January and February when the soil was covered with snow the fungal DNA concentration values were higher than in the samples from March when snow was melting away. Also other analysed parameters showed a similar tendency. The best examples are the number of CFF in Soil1 in March (Fig. 3a), the number of CFU of yeasts and maltose utilizing bacteria in Soil1 (Fig. 3c), the total soil DNA amount in Soil1 (Fig. 5a), fungal DNA amount in Soil1 (Fig. 5d), and H' in both experimental plots (Fig. 6b) in March. This tendency is slightly different in Soil2, which remained unfrozen during the winter. In this experimental plot the values of the analysed indicators continued to increase also during March and decreased only in April.

The DNA amount of *Trichoderma* spp. was similar at both sampling depths in the experimental plots, and did not differ significantly between the AVP and RP (Fig. 6). However, it was significantly ($F = 32.088$; $p = 0.015$) higher in Soil2 (3.64 ± 3.10 ng g^{-1} dry soil) than in Soil1 (0.72 ± 0.44 ng g^{-1} dry soil, except the values in March). Both experimental plots had on average a higher percentage of *Trichoderma* spp DNA of the total fungal DNA in the AVP compared to the RP, but these differences were statistically not significant (6.68% and 4.50% versus 1.81% and 1.43% in Soil1 and Soil2, respectively). *Trichoderma* spp. DNA was found also in soil samples in which no CFU of *Trichoderma* genus were detected.

In general the correlation between the results of the conventional plate count (number of CFF) and the amount of fungal DNA was weak (correlation coefficient 0.18). This observation may reflect the accumulation of unculturable or psychrophilic fungi that enjoy optimum growth conditions under the snow

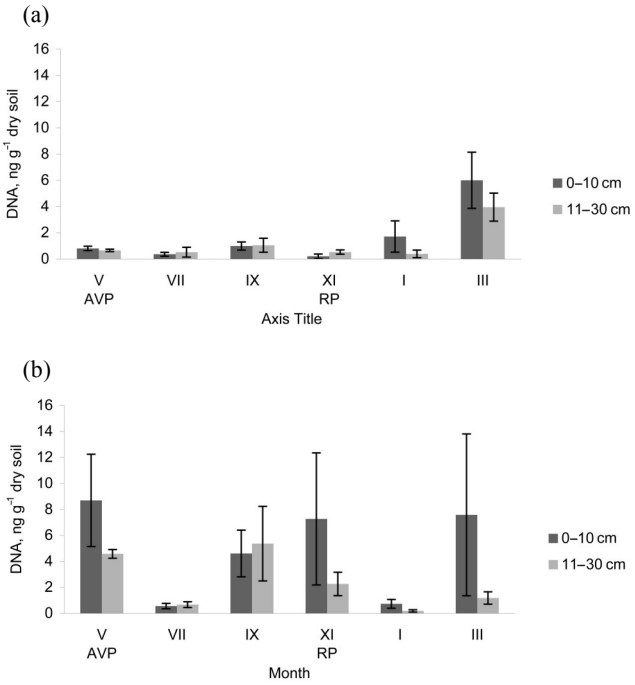

Fig. 6. Amount of *Trichoderma* spp. DNA estimated using qPCR in sampling plot Soil1 (a) and Soil2 (b) at both sampling depths (±SD; $n = 3$) in every second month.

cover in the presence of decaying plant litter. Their DNA could be amplified and identified, but we still could not grow the corresponding fungi in standard laboratory conditions.

Results of ARDRA

Typical results of ARDRA in 6% polyacrylamide gel electrophoresis are shown in Fig. 7 and changes of the Shannon–Weaver diversity indices of fungi in the experimental plots are given in Fig. 8. Fungal diversity indices were similar in both experimental plots and at both sampling depths. There were no statistically significant differences between the AVP and RP, but in some months (June, September, January) the fungal diversity was significantly lower than on average. The weak potential of molecular analysis derived diversity indices to evaluate the response of microbial populations to environmental factors has been recognized previously (Hartmann & Widmen, 2006). However, comparison of differences in the fungal community structure using cluster analysis (Fig. 9) showed tendencies for the soil samples from the RP to form a separate cluster in the case of Soil1 and several clusters in the case of Soil2. This means that the fungal communities were structurally different in the RP and AVP.

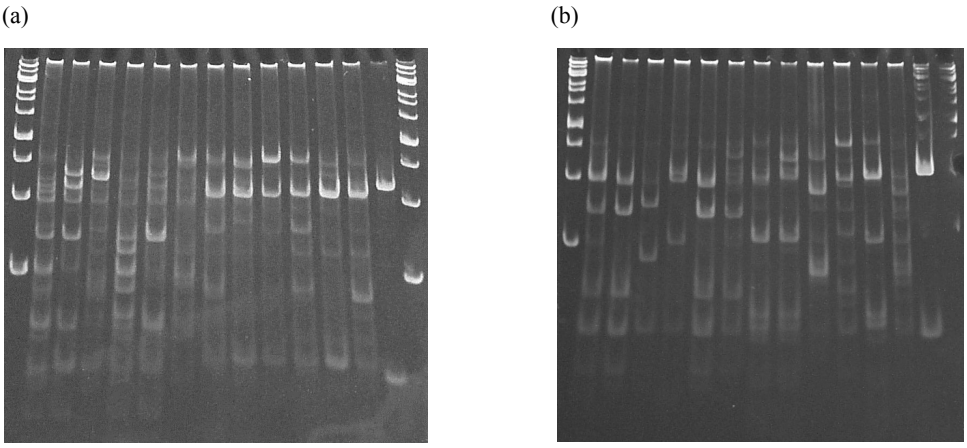

Fig. 7. Results of ARDRA in 6% polyacrylamide gel electrophoresis (August (a) and February (b)). Lanes 1–6 Soil1, 7–12 Soil2. +K, restriction products of *Heterobasidion parviporum* MSCL 981 DNA amplification. M, Gene Ruler 1 kb DNA Ladder (Fermentas).

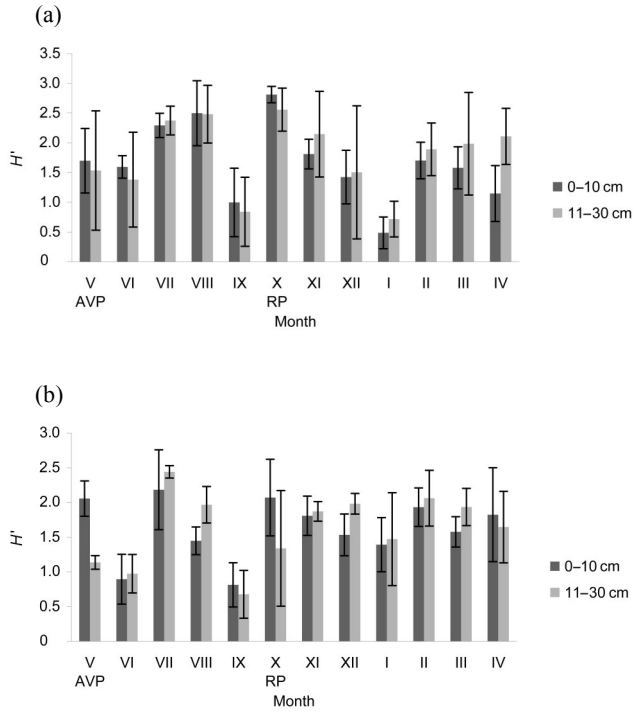

Fig. 8. Changes of the Shannon–Weaver diversity indices *H′* of fungi in sampling plots Soil1 (a) and Soil2 (b) at two depths (± SD; $n = 3$) in the investigated period. Indices were obtained using ARDRA in 6% polyacrylamide gels.

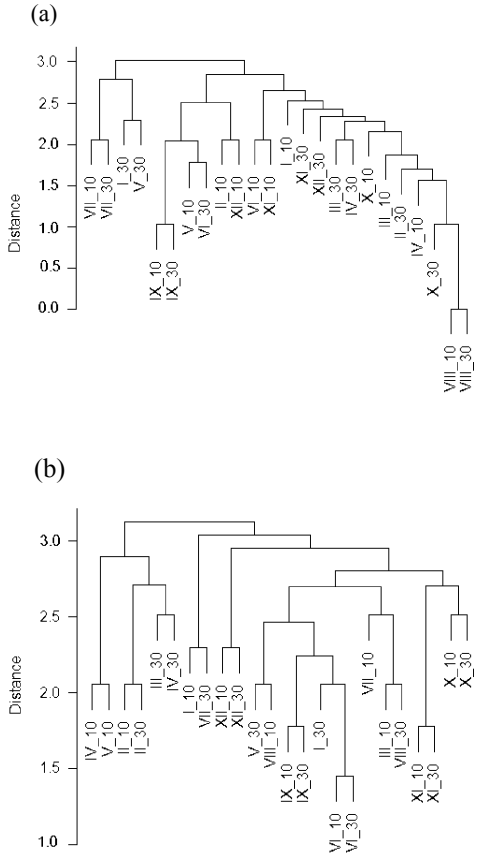

Fig. 9. Cluster analysis of seasonal fungal community structure differences in Soil1 (a) and Soil2 (b).

Results of the statistical analysis

In temperate regions the highest input of litter to the soil occurs in autumn when many plants are senescing and plant roots are dying. The winter microbial population starts to decompose the litter and to use phenolic carbon sources as well as to immobilize nitrogen. During snowmelt these populations die off and the accumulated nitrogen is used for plants and summer microbial populations. In cold systems the limiting factor for microbial growth is the availability of water but not the temperature. Under the snow even when the air temperature is significantly below 0 °C unfrozen water remains around the soil particles (Schmidt & Lipson, 2004).

Results of multiple regression analyses are listed in Table 4. According to these analyses, the number of CFF was significantly affected by soil moisture

Table 4. Results of multiple regression analyses

Factor	CFF	Yeasts and maltose utilizing bacteria	Total number of micro-organisms	Total amount of soil DNA	*H*′	Total amount of fungal DNA	Total amount of *Trichoderma* spp. DNA
Soil pH	NS	0.020[b]	0.005[b]	NS	NS	NS	NS
Average air temperature	0.01	NS	NS	0.020	NS	<0.001[b]	NS
Soil moisture content	<0.001	NS	NS	<0.001	NS	<0.001	<0.001
Multiple *R*-squared	0.387	0.459	0.489	0.551	–	0.537	0.627
p-Value of the model	<0.001	<0.001	<0.001	<0.001	–	<0.001	<0.001

[b] – negative impact; NS, not significant; –, not applicable.

content (Pearson correlation coefficient $r = 0.59$; $p < 0.001$) and to some extent also by average air temperature ($r = 0.23$; $p = 0.01$). When both experimental plots were analysed separately, the data showed no correlation between air temperature and the number of CFF in Soil1, but in Soil2 there was a weak correlation ($r = 0.48$; $p < 0.001$). The significant positive impact of soil moisture content on the number of CFF explains the low number of fungal isolates from Soil2 in July, October, and January: during these months the soil in this experimental plot was very dry, the structure of the soil (Table 2) facilitated drainage and did not allow accumulation of the water precipitated during previous months.

According to the results of multiple regression analyses, the impact of soil moisture content and average air temperature on the number of yeasts and maltose utilizing bacteria and also on the total number of culturable microorganisms was significant, and high soil moisture together with high average air temperature can explain the increased numbers of CFU obtained in August. When the soil moisture content was higher, we observed higher numbers of CFF as well as a higher total amount of the fungal DNA, including *Trichoderma* spp. DNA. The numbers of yeasts and maltose utilizing bacteria were not so affected by soil moisture as the CFF, with the exception of the combination of high soil moisture with high air temperature as in the case of Soil2 in August.

The soil pH varied in the analysed soil horizons from 2.42 to 3.68. In Soil2, which had a lower pH, the amount of yeasts and maltose utilizing bacteria was on average higher: 3.13×10^6 CFU g^{-1} of dry soil in the upper soil layer and 9.83×10^5 CFU g^{-1} of dry soil in the deeper soil layer versus 1.26×10^6 CFU g^{-1} of dry soil and 6.96×10^5 CFU g^{-1} of dry soil in Soil1. The soil pH, which was different between the experimental plots, significantly influenced the number of yeasts and maltose utilizing bacteria and the total number of soil microorganisms (data not shown). The total amount of soil DNA was influenced by the soil moisture ($r = 0.65$; $p < 0.001$) and average air temperature ($r = 0.22$; $p < 0.001$).

When data of the experimental plots were analysed separately, no correlation was found between the air temperature and soil moisture and the total amount of soil DNA in Soil1, while in Soil2 there was a weak correlation of the total amount of soil DNA with air temperature ($r = 0.42$; $p < 0.001$) and a moderately strong correlation with soil moisture ($r = 0.78$; $p < 0.001$). According to the results of *t*-test, these differences were not statistically significant, probably due to the fact that in Soil2 the CFU counts were significantly different between the AVP and RP.

Total amount of fungal DNA was significantly negatively affected by air temperature ($r = -0.60$; $p < 0.05$) (Fig. 10a) and positively affected by soil moisture content ($r = 0.44$; $p < 0.001$). Higher concentrations of fungal DNA at lower air temperatures were found in both experimental plots. The total amount of *Trichoderma* spp. DNA was affected only by soil moisture content ($r = 0.76$; $p < 0.001$) (Fig. 10b).

Fungal diversity expressed as the Shannon–Weaver diversity index H' was not influenced by any of the analysed factors.

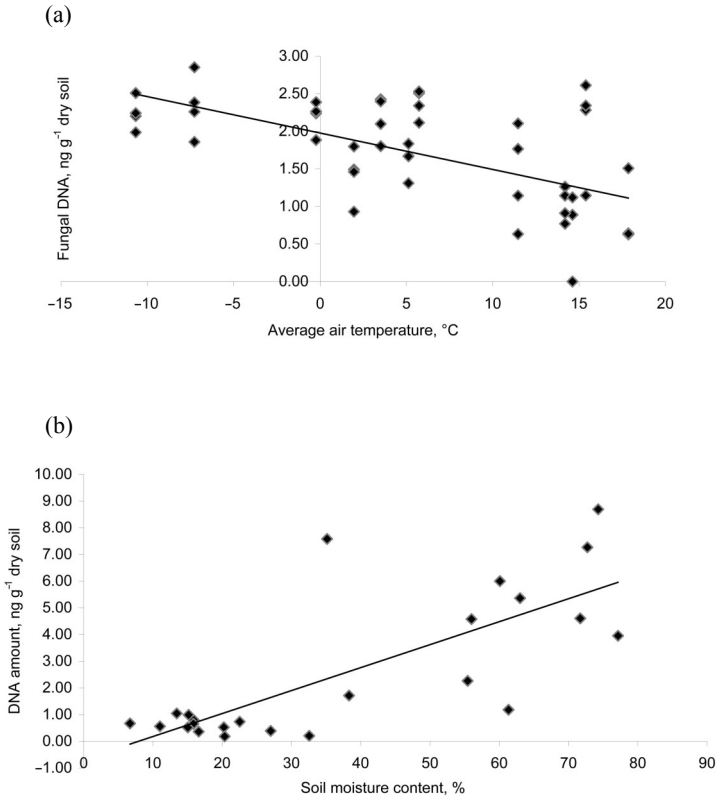

Fig. 10. Charts of correlations between average air temperature and total fungal DNA amount ($R^2 = 0.36$) (a) and between soil moisture content and *Trichoderma* spp. DNA amount ($R^2 = 0.58$) (b).

CONCLUSIONS

We can conclude that the microbial communities of both closely located experimental plots showed a different response to changing environmental factors during the AVP and RP. This can be explained by the fact that the air temperature and precipitation differed between the plots due to the differences in their micro-habitat topography: soil type, texture, composition of chemical elements, which influenced the changes of soil moisture content and properties of soil horizons.

In the sod-podzolic soil (Soil1) only the fungal DNA amount varied significantly during the analysed period: it was significantly higher in the RP in comparison to the AVP.

In the illuvial humus podzol (Soil2) the number of culturable filamentous fungi, yeasts, and maltose utilizing bacteria as well as the abundance of *Penicillium* spp. were significantly higher in the AVP but the fungal DNA amount was significantly higher in the RP.

Several fungal species (*Geomyces vinaceus*, *G. pannorum*, *Mortierella alpina*, *Trichosporon porosum*) isolated from the soil samples of the RP have been detected in arctic environments or recorded as growing at low temperatures such as 0 °C. Although all our isolates of these fungi were able to grow at 4 °C, they did not show any psychrotrophic growth characters.

Several species were typically isolated only in the AVP (*Pochonia bulbillosa*, *Scytalidium lignicola*, *Metarhizium anisopliae* var. *anisopliae*, and *Penicillium montanense*). From these species *M. anisopliae* var. *anisopliae* was not able to grow at 4 °C. In general the growth rate was genus and species dependent.

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Sesoonsed muutused parasvöötme kuusemetsa mulla mikroobikoosluses

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Käesoleva uurimistöö eesmärgiks oli uurida sesoonseid muutusi parasvöötme kuusemetsa mulla mikroobikoosluses, kasutades traditsioonilisi ja molekulaarseid mikrobioloogilisi meetodeid. Materjal mikrobioloogilisteks analüüsideks koguti mulla kahest ülemisest horisondist kahelt eri tüüpi mullaga hariliku kuuse alalt 12 kuu pikkuse perioodi jooksul. Mulla mikroobikooslus oli seotud erinevate keskkonnateguritega uuritud aladel. Vegetatsiooniperioodiga (maist septembrini) võrreldes oli leetmulla korral seente DNA kogus mullas oluliselt suurem puhkeperioodil (oktoobrist aprillini) ja *Penicillium*'i perekonna liikide arv oli suurem vegetatsiooniperioodil. Huumusilluviaalse leedemulla puhul oli pärmide, *Penicillium*'i perekonna liikide ja kultiveeritavate niitjate seente arvukus suurem vegetatsiooniperioodil. 84 seeneisolaadi ITS-i järjestuste analüüs näitas kahe uuritud ala seenekoosluse suurt erinevust (Sørenseni indeks 0,29), kuid sesoonne erinevus oli väiksem (Sørenseni indeks 0,48). Puhkeperioodil kogutud seeneisolaadid olid võimelised kasvama 4 °C juures, kuid ükski neist isolaatidest ei olnud psührotroofne tüvi.

3.3. Particular characteristics of soil microbial communities in forest stands infected with *Heterobasidion parviporum* and *Armillaria* spp.

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Particular characteristics of soil microbial communities in forest stands infected with *Heterobasidion parviporum* and *Armillaria* spp.

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We compared soil microbial communities of forests infected with *Heterobasidion parviporum* and *Armillaria* spp. with soils of healthy forests using conventional plating and molecular methods. Plate counts from the soils of the infected forests reflected a significant decrease of the number of cultivable filamentous fungi (CFF) and a slight decrease of the total number of cultivable microorganisms. The diversity of CFF was reduced in the stands infected with *H. parviporum*. In the stands infected with *Armillaria* spp. the diversity of CFF and relative abundance of cultivable *Trichoderma* spp. was even higher than in healthy forest stands. Quantitative PCR revealed increased concentrations of total fungal DNA and *Trichoderma s*pp. DNA in the soil of *H. parviporum* infected stands. In *Armillaria* spp. infected stands the total concentration of fungal DNA was decreased, but relative amount of *Trichoderma s*pp. DNA was increased. No significant differences in the species diversity of fungi in the soil were found by ARDRA.

Keywords: soil; forest; Shannon - Weaver diversity index; *Heterobasidion annosum; Armillaria*; *Trichoderma*.

1. Introduction

Several properties of forest site and soil affect the occurrence and severity of the damage of such serious tree root pathogens as *Heterobasidion annosum* and *Armillaria* spp. In general, a high risk of *H. annosum* damage has been associated with high fertility of the site, high pH and calcium concentration, but also low organic matter content and a relatively high content of sand in the soil favour infection [1 - 3]. The main reason is believed to be the lack of such antagonistic fungi as *Trichoderma* and *Penicillum* spp. [1].

The factors by which soils can be suppressive to different pathogens can involve biotic and/or abiotic elements, and they may be different with various pathogens. There are suggestions that the main agents in soil suppressiveness are microbial [4]. The importance of antagonistic microflora has been reported not only in the case of *H. annosum* but also regarding honey fungus *Armillaria* spp. It is described that rhizomorph formation and growth of particular *Armillaria ostoyae* isolates declined in the presence of particular isolates of *Trichoderma hamatum*, *T. viride*, *Mycelium radicis atrovirens α* and *Penicillium spinulosum* [5].

Root rot is common in Latvia and other countries of the northern temperate region. The identification of *H. annosum* intersterility groups in Latvia previously was done using mating compatibility tests [6].

The aim of this investigation was to create baseline information about particular characteristics of soil microbial communities of infected forest stands in comparison with healthy forests.

2. Materials and methods

From 40 % of trees in sampling plots Aa and Ab (sampling time May 2007) the root rot fungus *Heterobasidion annosum* (Fr.) Bref. was isolated. Signs of the decay were detected in 80 % of the trees. 30 % of the trees at sampling plots Ba, Bb and Bc (October 2006) were infected with honey fungus *Armillaria* sp. which was detected by the presence of rhizomorphs or characteristic, white mycelial mats under the bark. In the forests Ca, Cb and Cc (October 2007) *H. annosum* and *Armillaria* spp. were not detected. Properties of sampling plots are given in Table 1.

Soil samples in Aa, Ab, Ba, Bb and Bc plots were taken 0-10 and 10-30 cm deep. The sampling plot was a circle with three transects on which samples at each depth were taken at 0.3 m, 1.0 m and 3.0 m from the centre. In healthy forests two samples were taken using soil profiles or outcrops. The pH and water content of soil samples was estimated according to the methods ISO 10390 and ISO 11465, respectively.

From all forest stands several drilled wood samples were obtained with an increment borer and placed in sterile glass test-tubes. In the lab they were placed on agarized malt extract (Biolife) (15 g/l) and incubated at room temperature for 1-2 weeks. The mycelia of root rot fungus was identified microscopically and placed on

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Table 1 Sampling plots and their characterization.

Location	Soil profile	Soil type according to FAO WRB 2006 [7]	Dominate tree species	Soil pH $_{H2O}$*	Water content at each depth, %
Riga	Aa	Cutanic Stagnic Albeluvisols	Spruce (*Picea abies*)	4.38±0.26	28.4; 20.4
	Ab	Placic Rustic Albic Folic Stagnic Podzols	Spruce	5.12±0.42	70.0; 49.0
Jekabpils	Ba,	Histosols	Pine (*Pinus sylvestris*), birch (*Betula pendula*), aspen (*Populus tremula*), gray alder (*Alnus incana*)	4.98±0.39	76.1; 74.1
	Bb,	Histosols		4.65±0.28	13.7; 12.8
	Bc	Histosols		5.50±0.35	23.5; 18.2
Cesis	Ca	Haplic Cambisols	Spruce, pine, birch, gray alder	5.12±0.23	42.5; 8.4
Valka	Cb	Colluvic Cambisols	Spruce, pine, birch	5.30±0.53	6.6; 3.0
Valka	Cc	Haplic Arenosols	Pine monoculture	4.46±0.62	4.5; 2.9

* The average pH (±S.D.) calculated from the pH measurements of all analyzed depths

new plates. The DNA was extracted from these pure cultures and directly from the drilled wood using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc.). The type of root rot fungus was identified by PCR [8]. DNA from *H. annosum* strain MSCL 532 (isolated from infected pine) obtained from the Microbial Strain Collection of Latvia (MSCL) was used as the control. ITS1-5.8S-ITS2 rDNA region of the isolated and the reference strains was PCR amplified using primers ITS1F and ITS4 [9]. 5 µl of the amplified products were cycle sequenced at the Latvian Biomedical Research and Study Center using BigDye Terminator v3.1 Kit and with one of the two primers. Sequences were analyzed using Staden Package 1.6.0.

To estimate the number of colony forming units (CFU) of fungi, bacteria and yeasts three replicates of soil sample dilutions were analyzed. Agarized malt extract (30 g/l) was used for cultivation of fungi, yeasts and some groups of bacteria. Further in the text all these groups together are referred to as cultivable microorganisms. Incubation time was 120 h and temperature was 20 ± 2 ˚C [10].

Fungal genera were determined according to morphological characteristics using keys [11, 12]. For estimation of the Shannon-Weaver diversity index for the genera of CFF (H'_{CFF}) the following equation was used:

$H'_{CFF} = -\sum p_j \log_2 p_j$, where p_j – relative abundance of particular genera of CFF.

From eleven colonies of sterile mycelia DNA was extracted using the same soil DNA isolation kit and these cultures were subjected to sequencing as described previously.

Total soil DNA was extracted with PowerSoil™ DNA Isolation Kit. Samples were homogenized using horizontal Mixer Mill Type MM 301 (Retsch) at a maximal speed of 30 Hz for 10 minutes. DNA was pooled from two replicates of the soil sample. Total amount of the soil DNA was determined spectrophotometrically (Ultrospec 3100 Pro, Amersham Biosciences).

Amplified rDNA restriction analysis (ARDRA) was performed using universal fungal primers ITS1F and ITS4. These primers amplify the nuclear rDNA region of *Ascomycota, Basidiomycota* and *Zygomycota* [13]. The reactions contained 1 µl of DNA template, 2 u of Hot Start *Taq* DNA Polymerase, 5 µl 10X Hot Start PCR Buffer, 5 µl dNTP Mix, 2 mM each, 4 µl 25 mM $MgCl_2$ (Fermentas), 1 µl of each 25 µM primer (OPERON Biotechnologies), and sterile distilled water to 50 µl. The PCR conditions: 4 min at 95 ºC, (40 s 95 ºC, 40 s 52 ºC, 1 min 72ºC) x 30 and 10 min at 72 ºC.

Amplified PCR products were digested with the restriction endonucleases *Bsu*RI or *Eco*RI [14]. Restriction products where run in 2 % agarose gels; photographed and analyzed with KODAK1D software. For estimation of the Shannon–Weaver diversity index (H') the following equation was used:

$H' = -\sum p_j \log_2 p_j$, where p_j – relative intensity of individual band [15] summing up results of both restriction nucleases [16].

To determine relative abundance of the total fungal DNA and DNA in soil we performed quantitative PCR using universal primers ITS1F and ITS4 for ITS1-5.8S-ITS2 rDNA region of fungi or uTr and uTf primers for *Trichoderma* spp. [17]. Calibration curve for *Trichoderma* spp. was built using DNA from pure culture of *Trichoderma harzianum* MSCL 309. Calibration curve for total soil fungal DNA was calculated as an average from four species specific DNA curves, which were obtained using universal primers ITS1F and ITS4 and DNA from pure cultures of the fungi *H. annosum* isolated from the trees of sampling plot A1, *Penicillium lanoso-viride* MSCL 1, *Trichoderma harzianum* MSCL 309 and *Mucor* sp. MSCL 15. Each DNA sample was analyzed in three replicates. The reactions contained 1 µl of the DNA template, 12.5 µl SYBR® Premix Ex Taq™ (TaKaRa), 1 µl of each 25 µM primer and sterile dH_2O to 25 µl. PCR conditions on SmartCycler (Cepheid): 60 s 95 ºC; (30 s 95 ºC, 30 s 55 ºC for primers ITS1F and ITS4 or 60 ºC for primers uTr and uTf, 30 s 72 ºC) x 40.

For statistical analyses F-test, t-test (α=0.05) and correlation analyses with *Excel* were done. Program R was used for multiple regression (α=0.05) analyses.

2. Results and discussion

We used two methods to identify the species of root rot fungus *Heterobasidion annosum* (Fr.) Bref. *sensu lato*. The results of PCR reactions (in Fig. 1) show that the forest stands in the sampling plot Aa and Ab were infected with root rot fungus *H. parviporum* (Fr.) Niemelä and Korhonen and that it was possible to identify the root rot fungus type using both – DNA isolated from the pure cultures of the pathogen and DNA extracted directly from the infected wood. According to the PCR results the isolate MSCL 532 was *H. annosum* (Fr.) Bref. *sensu stricto*.

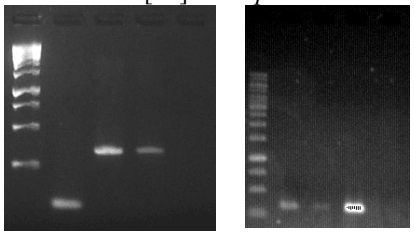
In addition, the fungal rDNA region of the isolate from sampling plot Aa (accession no. GU296434) had 99% similarity to the ITS sequences of *H. parviporum* and 100 % similarity although with less query coverage with several sequences named as *Heterobasidion abietinum*. The explanation is that sequences of the ITS of *H. abietinum* differ only by two residues from the major *H. parviporum* sequence variants [18]. Since fungus *H. abietinum* has not been found in Eastern and Northern Europe [19] we can assume that our isolate is *H. parviporum*. The isolate from the sampling plot Ab (accession no. GU296435) has 99 % similarity to the ITS sequences of *H. parviporum* and with several sequences named as *H. annosum* [18]. We can take into consideration these last mentioned sequences but they were published before 1998 when the intersterility groups occurring in Europe were named as *H. parviporum* and *H. annosum sensu stricto* [20]. Isolate MSCL 532 (accession no. GU296436) has 99 % similarity to the ITS sequences of *H. annosum*.

In all sampling plots there is a tendency that with increasing depth the number of CFF significantly decreases (Fig. 2a). The tendency holds also for a portion of soil profiles (Aa, Ab, Ca and Cc) if we consider the total number of cultivable microorganisms (in Fig. 2b). The decrease of the quantities of cultivable microorganisms within depth in different soils as well as in forest soil has been widely recorded [21, 22].

Plots infected with *H. parviporum* on average have lower numbers of CFF and higher total numbers of cultivable microorganisms in comparison with sampling plots infected with *Armillaria* spp.. That means that soils in sampling plots Aa and Ab have higher numbers of bacteria and/ or yeasts. In comparison soils of healthy forests have increased numbers of both CFF and cultivable microorganisms, especially in the upper soil horizon.

Identified CFF belonged either to *Ascomycota* or *Zygomycota*. In all soil samples representatives from genera *Penicillium, Trichoderma* and *Mucor* were found. Only in infected stands representatives from genera *Aureobasidium, Cladosporium, Cylindrocephalum, Fusarium, Neurospora, Staphylotrichum* and *Rhizopus* were detected. *Geomyces* spp. was found only in healthy forests. As mentioned in the literature the incidence of root rot is correlated with the lack of such antagonistic fungi as *Trichoderma* and *Penicillum* spp. [1] but the soil from the sampling plots infected with root rot contained as high numbers of *Penicillium* (9 % from all detected fungi) as healthy forests, only the amount of Trichoderma spp. was significantly reduced (0.5 % in comparison with 1.5 % in healthy forest soil). Similar results were obtained in other investigations in Latvia [23]. Stands infected with *Armillaria* spp. have higher proportion of *Penicillium* spp. (15-40 %) and *Trichoderma* spp. (2-10 %) in comparison with other sampling plots (less than 10 %). It is reported that some *Penicillium* species may stimulate the growth of *Armillaria ostoyae* [5].

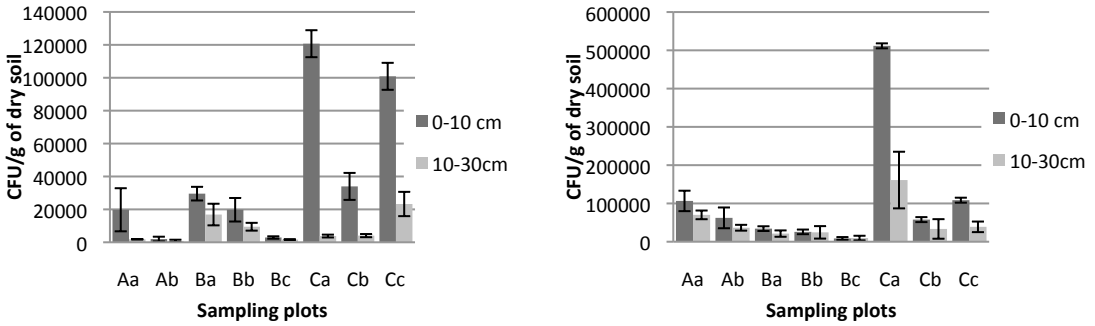
Sterile mycelia isolates were abundant in all the sampling plots. In stands Aa and Ab sterile mycelia made up 25–90% of all CFF colonies, while in sampling plots Ba, Bb and Bc – 5–35 % of all CFF colonies. In healthy forest stands sterile mycelia constituted on average 65 – 95 % of CFF colonies. The molecular analysis of 11 sterile mycelia identified three of them (all from sampling plot Ab) as *Basidiomycota* (*Fomitopsis pinicola*, *Gloeophyllum sepiarium,* and uncultured fungus clone) four as *Ascomycota* (*Lecythophora mutabilis* from plot Ab; *Tolypocladium cylindrosporum* and *Beauveria geodes* from plot Ca; *Helotiales* from plot Cc) and other four as *Zygomycota* - *Mortierella sp.* (Aa, Ab), *Umbelopsis* sp. (Aa) and *Mucor hiemalis* (Aa). *F. pinicola* is generally regarded as saprophytic basidiomycete but it may cause heart-rot disease in living tissues of birch [24] and other trees [25]. *G. sepiarium* is a secondary colonizer of wood, causative agent of brown rot [25].



a) b)

Fig. 1 PCR fragments amplified with primer set MJ-F, MJ-R, KJ-F and KJ-R. a) 1- Marker Gene Ruler 1 kb DNA Ladder (Fermentas). 2. *H. annosum* MSCL 532; 3., 4. PCR amplification products of root rot fungi isolated from the infected trees in sampling plots Aa and Ab (respectively); 5. Negative control. b) 1- Marker. 2., 3. PCR amplification products of DNA isolated directly from the pieces of infected trees in sampling plots Aa and Ab (respectively); 4. DNA of *H. parviporum* (identified in the gel A). 5. Negative control.

Shannon-Weaver diversity indexes H'_{CFF} are shown in Fig. 3. Soil in sampling plots Ba, Bb and Bc has higher diversity in comparison with other soils, especially in deeper soil layers. In sampling plots Aa, Ab and Ca the differences between two soil depths are statistically significant. The highest diversity of CFF and yeasts in the upper layer (0-15 cm) of soil in comparison with other soil depth layers was found also in the similar study in *Salix* sp. and *Populus* sp. forest in Austria [22].



a) b)

Fig. 2 The number of CFU of soil microorganisms. a) The number of filamentous fungi (means ± S. D.). b) Total number of cultivable microorganisms (means ± S. D.).

Shannon-Weaver diversity indexes H' are shown in Fig. 4. There are no statistically significant differences among samples from different soil depths and among all sampling plots. Other studies using ARDRA show that fungal diversity in forest soils tend to decrease in deeper layers [26].

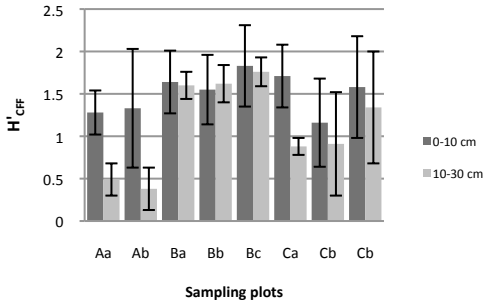


Fig. 3 Shannon-Weaver diversity indexes H'_{CFF} (means ± S. D.).

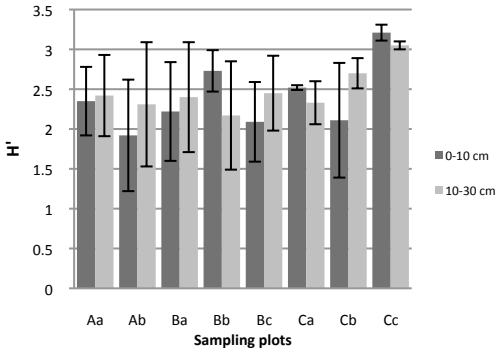


Fig. 4 Shannon-Weaver diversity indexes H' (means ± S.D.).

Sampling plots infected with *H. parviporum* and healthy forest stands have similar amounts and distribution of fungal DNA throughout the soil profile. More DNA can be detected in the upper horizons: $(9.51\pm1.46)\times10^{10}$ copies/g of dry soil (±S.D.) in the infected plots Aa and Ab; $(5.52\pm3.91)\times10^{10}$ copies/g of dry soil in healthy plots. Less DNA is found in the deeper horizons: $(6.49\pm1.25)\times10^{10}$ copies/g of dry soil in the plots Aa and Ab; $(5.06\pm1.5)\times10^{10}$ copies/g in the healthy forests. Fungal DNAs make up 4.48–8.66 % of total soil DNA. Stands infected with *Armillaria* spp. have similar amounts of fungal DNA in both soil layers: $(5.14\pm1.97)\times10^{10}$ copies/g of dry soil, or 2.64 – 4.49 % of the total soil DNA. Healthy forest stands have significantly less *Trichoderma* spp. DNA in comparison with infected ones: $(4.09\pm3.49)\times10^{8}$ copies/g of dry soil or 0.59 – 0.84 % from total fungal DNA in healthy stands *vs*. $(8.17\pm6.04)\times10^{8}$ – $(16.10\pm7.12)\times10^{8}$ copies/g of dry soil and $(8.55\pm6.45)\times10^{8}$ – $(9.21\pm7.49)\times10^{8}$ copies/g of dry soil, or 1.10 – 2.59 % of DNA in infected stands. The contradictory results with the plate count method in the case of *H. parviporum* infected stands can be explained by possible suppressive growth effect of other CFF in this method. 1.2×10^{6} copies /g fresh soil of *Trichoderma* spp. DNA were recorded in the beech forest soil in Southern Germany [17].

Multiple regression analysis demonstrated that the number of CFF negatively correlates with the sampling depth (p=0.005) and positively correlates with the soil moisture content (p=0.0009), average air temperature at the sampling day (0.045) and with the infection of the trees by pathogenic fungi (p=0.001). R^2 of the model was 0.85, p=0.0002. Total number of cultivable microorganisms positively correlates with soil moisture (p=0.002), average air temperature (p=0.000022) and by the infection of the trees (p=0.000016). R^2 of the model was 0.81, p=0.0002. Diversity of CFF positively correlates with the sampling depth (p=0.01), average air temperature (p=0.0008) and with the infection of the trees (p=0.007). R^2 of the model was 0.73, p=0.001. The infected forest soil in comparison with healthy one contain more *Trichoderma* spp. specific DNA (p=0.02). R^2 of the model was 0.47, p=0.02. Other analyzed parameters were not significantly affected by any of the factors included in regression models. There is no significant impact of the soil pH to the results.

Forest soil ecosystem is very complicated and it is difficult to evaluate all the interactions, such as relationship among serious forest pathogens (*H. parviporum* and *Armillaria* spp.) and other fungi inhabiting soil and trees. Nevertheless the comparative analysis of fungal communities in soils under infected natural forests has provided important baseline information about fungal diversity and composition in these ecosystems.

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3.4. Features of saprophytic soil microorganism communities in conifer stands with or without *Heterobasidion annosum sensu lato* infection: a special emphasis on *Penicillium* spp.

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Features of saprophytic soil microorganism communities in conifer stands with or without *Heterobasidion annosum sensu lato* infection: a special emphasis on *Penicillium* spp.

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Abstract

The aim of the study was to characterize soil microbial communities of nine conifer (*Pinus sylvestris, Picea abies*) stands mainly on podzolic soils, e.a., seven healthy stands (28 to 160 years) and two stands infected with root rot fungus *Heterobasidion annosum s.l.* (50 and 56 years). The studied microbial variables differed for healthy conifer stands on automorphic soils vs. infected conifer stands on semihydromorphic soils, and differed depending on forest age. Automorphic soils had significantly higher numbers of filamentous fungi in the upper soil layert han in the deeper layer. In semihydromorphic soils both analyzed soil layers had similar numbers of fungal colony-forming units. The pine stand had significantly lower yeast and maltose-utilizing bacteria density in the 11 to 30 cm soil layer. Sampling plots on semihydromorphic soils including infected stands lacked species of the *Mortierella* genus or had them in low percentage, but had increased percentage of colony-forming units of *Umbelopsis* genus. Semihydromorphic soils had decreased proportion of colony-forming units of *Penicillium* spp. and almost no *Trichoderma* spp. Molecular biology analysis showed that the average fungal diversity indice *H'* in automorphic soils was significantly higher than in semihydromorphic soils. The highest fungal diversity in respect to represented genera was in the oldest sampling plot (16 genera). Antagonism assay of 52 *Penicillium* spp. isolates showed reduced growth rate of *H. annosum s.l.* but the antagonistic effect was not as strong as it has been reported for *Trichoderma* spp.

Key words: antagonism assay, *Heterobasidion annosum, Mortierella, Penicillium*, soil microbial diversity, *Umbelopsis*.
Abbreviations: ARDRA, amplified ribosomal DNA restriction analysis; CFU, colony-forming units; MEA, malt extract agar; MSCL, Microbial Strain Collection of Latvia; PCR, polymerase chain reaction; *s.l., sensu lato; s.s., sensu stricto*.

Introduction

Soil in general is a very heterogeneous environment and the components of the solid fractions in soil such as sand, silt, clay, and organic matter provide variable microhabitats for the soil microorganisms (Garbeva at al. 2004). The most important soil parameters are particle size, pH, cation exchange capacity and organic matter content. These parameters can affect microbial community structure either directly by providing a specific habitat that selects specific microorganisms, or indirectly by affecting plant root functioning and exudation in a soil-specific manner. In some situations the soil and in others plant type is the determining factor affecting the soil microbial community (Garbeva et al. 2004).

Forest soils differ from agricultural soils by their vertical stratification due to the forest litter accumulation that forms the humic horizon, and saprotrophic soil fungi are one of the main groups of soil microorganisms responsible for litter degradation (Zeller et al. 2007; Baldrian et al. 2010; Allison, Treseder 2011). Their extracellular enzymes are active not only in the litter layer but also in the deeper soil horizons (Baldrian et al. 2010).

It is reported that several properties of site and soil also affect the occurrence and severity of the damage of such serious tree root pathogen like *Heterobasidion annosum sensu lato* (s.l.), which is widely found in Latvia (Grantina et al. 2000) and in other countries of the boreo-nemoral region. In general, high risk of *H. annosum* damage has been associated with environmental conditions and climate zones that determine soil properties and soil types. According to Pratt et al. (2002), *H. annosum* hazard increases with increase of site fertility (mainly on well drained calcareous soils with high pH value). However, hazard decreases with increase of soil moisture conditions (surface water gley and groundwater gley features) and accumulation of peat. According to Kaarna-Vuorinen (2000) the relative frequency of butt rot is higher on drained peatland sites than on undrained peatland sites. The incidence of disease is also reported to be higher in mineral soils with a fluctuating groundwater table (Thor 2005). In addition, ditching of mineral soil sites increases the risk of *H. annosum* butt rot damage, possibly because the root systems suffer from occasional drought (Korhonen et al. 1998).

High incidence of root rot is correlated with lack of

antagonistic fungi like *Trichoderma* spp. and *Penicillum* spp. (Korhonen, Stenlid 1998; Stenlid, Redfern, 1998). Besides several species of *Trichoderma* genus that have been screened for their antagonism against *H. annosum s.l.* (Nikolajeva et al. 2012) other soil microfungi have been reported to show antagonistic properties against *H. annosum s.l.* For example, several fungal isolates from 25 to 45 years old spruce stands from *Myrtillosa mel., Hylocomiosa, Oxalidosa* forest types have shown antagonistic abilities against the S and P group of *H. annosum s.l. in vitro*. These were members of the genera *Aureobasidium* (only against S group), *Geotrichum*, some isolates from the genus *Mortierella, Mucor, Mycogone, Oidiodendron, Penicillium, Rhizopus, Trichoderma, Trichosporon*, and partly *Verticillium* (Arhipova et al. 2008). A similar spectrum of fungal genera with antagonistic properties were found in a later investigation of this research group on the microflora of the spruce rhizosphere in stands established on former agricultural lands and forest lands (Gaitnieks et al. 2009). From the forest soils of Scots pine (*Pinus sylvestris*) stands in Poland also several fungi have been isolated showing antagonistic properties against the *H. annosum* P group – *Absidia glauca, Gliocladium viride, Mortierella isabellina* (part of isolates), *Oidiodendron tenuissimum, Penicillium daleae, Penicillium janczewskii, Penicillium spinulosum, Penicillium purpurogenum, Penicillium waksmanii, Penicillium decumbens, Rhizopus oryzae, Thysanophora penicillioides, Trichoderma koningii, Trichoderma harzianum, Trichoderma viride, Trichoderma atroviride,* and *Verticillium* sp. (Manka et al. 2006).

The factors by which soils can be suppressive to different soil-borne pathogens can involve biotic (soil microflora) and/or abiotic elements (soil physical and chemical properties), and their impact may be different on various pathogens. It has been suggested that the main agents in soil suppressiveness are microbial (Garbeva et al. 2004).

In this investigation microbial communities in soils of healthy forest stands will be compared with those in soils of forest stands that are infected with pathogenic fungus *H. annosum s.l.* The aim of this investigation was to create baseline information about particular characteristics of soil microbial communities depending from soil group and forest age and to evaluate the antagonistic abilities of particular soil filamentous fungi against *H. annosum s.l.* The objectives of the study were to characterize and compare soil microbial communities using following methods: enumeration of cultivable microorganisms; determination of typical fungal genera and species using molecular biology methods; extraction of total soil DNA; polymerase chain reaction (PCR) followed by amplified ribosomal DNA restriction analysis (ARDRA) and calculations of Shannon-Weaver diversity indices; estimation of antagonistic abilities of 52 *Penicillium* spp. isolates from agricultural soil, former agricultural soil and forest soil against two isolates of *H. annosum sensu stricto* (*s.s.*), one isolate of *H. annosum s.l.* and two isolates of H*eterobasidion parviporum*.

Materials and methods

Sampling plots, soil and wood sampling

Sampling plots were located in the Ropaži district of Latvia. These forest stands are under the management of Riga Forests Ltd. Characteristics of each sampling plot are described in Table 1. From all forest stands 20 wood samples were obtained with increment borer and placed in sterile glass test-tubes. Where present fruiting bodies of *H. annosum s.l.* were sampled (sampling plot Spruce 50).

Soil samples (500 g) were taken in two depths (0 to 10 cm and 11 to 30 cm) using a modified method described by Luis et al. (2005). Sampling plots were circular with three transects along which three samples in each depth

Table 1. Sampling plots, their characterization and sampling time. Forest types are according to the classification of Buss (1997). [a], Automorphic soils – soils without gleyic or gley horizons. The formations of gley can be observed only as separate pathces under the organic horizon and/or as obstructions in gaps and tunnels of earthworms. [b], Semihydromorphic soils – seen only with standing water or medium deep groundwater (Karklins 2008)

Sampling plot		Sampling date	Forest age (years)	Soil type according to Latvian soil classification (Karklins 2008)	Soil group according to FAO WRB 2006
Healthy forest stands					
Pine 80	*Myrtillosa*	June 2010	80	Typic podzol[a]	Folic Arenosols
Spruce 37	*Oxalidosa*	August 2010	37	Illuvial humic podzol[a]	Folic Albic Podzols
Spruce 47	*Oxalidosa*	July 2010	47	Stagnogley sod-podzolic soil[b]	Folic Gleyic Calcic Luvic Planosols
Spruce 28	*Hylocomiosa*	June 2010	28	Illuvial humus podzol[a]	Podzols
Spruce 62	*Hylocomiosa*	June 2010	62	Typic podzol[a]	Folic Albic Podzols
Spruce 141	*Hylocomiosa*	August 2010	141	Typic podzol[a]	Folic Podzol
Spruce 160	*Vacciniosa*	June 2010	160	Sod-podzolic soil[a]	Folic Arenosols
Infected forest stands					
Spruce 50	*Oxalidosa*	July 2010	50	Humi-podzolic gley soil[b]	Rustic Folic Endogleyic Podzols
Spruce 56	*Hylocomiosa*	July 2010	56	Humi-podzolic gleyic soil[b]	Cutanic Albic Gleyic Luvisols

were taken, at 0.3 m, 1.0 m and 3.0 m from the centre. Samples were placed in sterile plastic bags (Nasco WHIRL-PAK), stored at 4 ^{0}C for a few days until the estimation of cultivable microorganisms and then were stored at –20 ^{0}C. In total 54 soil samples were analyzed.

Physical and chemical analysis of soil

Soil moisture content was determined according to the ISO 11465 standard method for every soil sample. The pH of soil samples was measured in distilled water according to the ISO 10390 method. Soil physical and chemical analysis was performed in the Laboratory of Forest Regeneration and Establishment Group of Latvian State Forest Research Institute „Silava". The following methods were used: LVS ISO 11464 (2006) Soil quality – Pretreatment of samples for physico-chemical analysis; LVS ISO 11465 (2006) Soil quality – Determination of dry matter and water content on a mass basis – Gravimetric method; LVS ISO 11277 (2010) Soil quality – Determination of particle size distribution in mineral soil material – Method by sieving and sedimentation; LVS ISO 11261 (2002) Soil quality – Determination of total nitrogen – Modified Kjeldahl method; LVS ISO 10694 (2006) Soil quality – Determination of organic and total carbon after dry combustion (elementary analysis); LVS ISO 10693:1995 Soil quality – Determination of carbonate content – Volumetric method; LVS ISO 11466:1995 Soil quality – Extraction of trace elements soluble in aqua regia; and LVS 398 (2002) Soil quality – Determination of total phosphorus. The content of humic acids was determined according to the method of Zaccone et al. (2009). From every plot four soil samples in three chemical replicates were analyzed.

Estimation of number of microorganisms and determination of dominant fungal genera

These parameters were determined in every soil sample in three replicates. In order to estimate the amount of colony forming units (CFU) of filamentous fungi, bacteria and yeasts soil sample decimal dilutions were prepared using sterile ddH_2O. Agarised malt extract (MEA, Biolife, Italy, 30 g L^{-1}, pH 5.4 ± 0.2) was used as growing medium, as it is favorable for filamentous fungi, yeasts and particular groups of bacteria, for example, actinobacteria. Further in the text all these groups together are referred to as cultivable microorganisms. Incubation time was three days for the enumeration of bacterial CFU and five days for the enumeration of fungal CFU, and temperature of incubation was 20 ± 2 °C. Fungal genera were determined according to morphological characteristics and light microscopy results using keys.

Detection of root rot fungus in wood samples

In the laboratory, collected pieces of wood were placed on the MEA (15 g L^{-1}) and incubated at room temperature for 1 to 2 weeks. The mycelia of root rot fungus was identified microscopically and placed on new Petri dishes with MEA. Forest stands from which no isolate of *Heterobasidion* sp. was obtained and no fruiting bodies were detected on the site, and also trees lacked any signs of infection, were further considered as healthy forest stands.

Extraction of total soil DNA, PCR and ARDRA

Total soil DNA was extracted using a PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc.). Samples were homogenized using a horizontal Mixer Mill Type MM 301 (Retsch) at maximal speed 30 Hz (1800 oscillations min^{-1}). The amount and purity of the DNA was detected spectrophotometricaly using Ultrospec 3100 Pro (Amersham Biosciences). In PCR we amplified the fungal nuclear ribosomal DNA region that contains two internal transcribed spacers and the 5.8S rRNA gene (ITS1-5.8S-ITS2) with primers ITS1F (Gardes, Bruns 1993) and ITS4 (White et al. 1990). These primers amplify the ITS1-5.8S-ITS2 region of *Ascomycota, Basidiomycota* and *Zygomycota* fungi. The PCR conditions and ARDRA methodology was used as described previously (Grantina et al. 2012; Grantina-Ievina et al. 2012). For ARDRA analysis the restriction endonuclease *Bsu*RI (Chabrerie et al. 2003) was used. For the calculation of the Shannon-Weaver diversity index (*H'*) the following equation was used:

$$H' = -\Sigma\, p_j \log_2 p_j,$$

where p_j is a relative intensity of individual band (Gabor et al. 2003).

Sequencing of ribosomal DNA

In total, 59 isolates (53 sporulating and six white sterile mycelia, not sporulating while kept at 4 °C for several months) representing dominant filamentous fungi were isolated from the plates used for the enumeration of cultivable filamentous fungi and subcultured on MEA. Genomic DNA from approximately 0.25 g of mycelia was extracted using a PowerSoil™ DNA Isolation Kit. Extracted DNA was amplified by PCR with primers ITS4 and ITS1F. Amplified DNA fragments from fungal isolates were sequenced in the CBS KNAW Fungal biodiversity centre, Utrecht, the Netherlands in the frame of project EMbaRC. After PCR, 5 µL of amplified products were subjected to a sequencing protocol with a BigDye Terminator Cycle Sequencing Kit (version 3.1, Applied Biosystems, USA) with both primers. Double stranded sequences of PCR amplicons were assembled using Staden Package 1.6.0. Homology search in the National Centre for Biotechnology Information (NCBI) GenBank nucleotide database was conducted using the Basic Local Alignment Search Tool.

Antagonism assay

In total, 52 *Penicillium* spp. isolates (Apendix 1) from agricultural soil, former agricultural soil (aspen stands) and forest soil including eight isolates from the present study and two isolates from spruce stands infected with

H. parviporum from the previous investigation (Grantina et al. 2012) were used in antagonism tests on 3 % MEA against two isolates of *H. annosum s.s.* (strain number in Microbial Strain Collection of Latvia MSCL 1020, MSCL 1021), one isolate from the sampling plot S56 *H. annosum s.l.* and two isolates of *H. parviporum* (MSCL 980, MSCL 981). Incubation temperature was 20 ± 2 °C. Each pairing with root rot isolates was performed twice. Petri dishes were checked and colonies measured three times or more until complete overgrowth of the dish had occurred (approximately 30 days). The efficiency of *Penicillium* in suppressing radial growth was calculated using growth measurements after 7 to 9 days of incubation as follows:

$$(C - T) / C \times 100,$$

where C is radial growth measurement of the pathogen in the control and T is radial growth of the pathogen in the presence of *Penicillium* (Asran-Amal et al. 2005).

Statistical analysis

For statistical analysis the *F*-test, *t*-test (α = 0.05), and correlation analysis with Excel function CORREL were performed. Multiple regression analysis was done with the R package (R Development Core Team 2009). Due to co-linearity of total carbon content values and calcium, magnesium and potassium values, C/N ratios were used in the regression models instead of total carbon content and nitrogen content.

Results and discussion

Physical and chemical characterization of soil

Data on element content, soil pH and soil moisture content are listed in Table 2. Soil pH_{H2O} was similar in all sampling plots and ranged from 3.71 to 5.08. The concentration of all analyzed elements significantly differed between both analyzed soil depths. The concentrations of several elements were significantly higher in semihydromorphic soils. For example, at depth 0 to 10 cm, concentrations of calcium (F = 2.00, p = 0.02), magnesium (F = 5.73, p = 0.008), potassium (F = 2.81, p = 0.02), carbon (F = 8.57, p = 0.04) and nitrogen (F = 3.54, p = 0.042) were significantly higher. At depth 11 to 30 cm, significantly higher levels of magnesium (F = 2.62, p = 0.02) and potassium (F = 1.97, p = 0.003) were observed.

The average soil moisture content of semihydromorphic soils was higher than of authomorphic soils, 54.07 ± 15.19% vs. 37.10 ± 21.52%, although this difference was not statistically significant.

Regarding soil texture, in all sampling plots the 0 to 10 cm sample contained only organic matter. At depth of 11 to 30 cm, the soil texture class was sand in sampling plots Pine 80, Spruce 62 and Spruce 160 and loamy soil in sampling plot Spruce 56. In other sampling plots the soil in this depth contained only organic matter.

Presence of root rot fungus in wood samples

Mycelium of *H. annosum s.l.* was isolated only from wood samples of sampling plot S56. Infection of root rot was observed only in two sampling plots, S50 and S56.

Amount of cultivable microorganisms and dominant fungal genera

The average numbers of CFU of filamentous fungi are given in Fig. 1. There was a clear difference between the two soil groups, automorphic and semihydromorphic soils. In sampling plots with automorphic soils the numbers of fungal CFU significantlt differed between soil depths. In the upper soil layer there were more filamentous fungi. In the sampling plots with semihydromorphic soils, which include two infected forest stands, the soil layers had similar number of fungal CFU. There were no differences between the soil of pine stand and spruce stands.

Significant differences in number of CFU of yeasts and maltose utilizing bacteria (Fig. 2) between dryer and moister soil types were not observed. In almost all sampling plots (with exception plot Spruce 50) there were significantly higher numbers of CFU of yeasts and maltose utilizing bacteria in the upper soil horizon. The pine stand had significantly lower number of yeasts and maltose utilizing bacteria, in comparison with spruce stands, in the deepest soil layer (11 to 30 cm; F = 21.89, $p < 0.001$), which can be explained by higher pH value (5.08).

The decrease of the abundance of microorganisms with depth has been previously reported, for example, in two soil profiles covered by annual grasses as vegetation in a Mediterranean climate (Fierer et al. 2003), or in forest soil profiles in Italy (Agnelli et al. 2004) and in deciduous woodland in UK (Castellazzi et al. 2004).

Regarding observed differences between the two soil moisture classes (automorphic and semihydromorphic soils), it has been reported that hydromorphic soils have higher yeast abundance than mineral soils (Polyakova et al. 2001) and that soddy-gley hydromorphic forest soils have high numbers of hydrolytic bacteria in comparison with peaty-podzolic and peat soils (Dobrovolskaya et al. 2000).

Abundances of the main fungal genera and sterile mycelia are given in Table 3. There were clear differences in the composition of the dominant fungal genera between automorphic and semihydromorphic soil classes. Sampling plots with semihydromorphic soils (Spruce 47, Spruce 50, Spruce 56) including infected stands, lacked species of *Mortierella* genus or their relative abundance of CFU was low (in average 0.67 %), but had higher percentage of CFU of the *Umbelopsis* genus (in average 40.66 %). These two genera are close relatives, and formerly the genus *Umbelopsis* with coloured colonies belonged to one section of the *Mortierella* genus. They both produce arachidonic acid, whitch acts as a suppressive agent of plant pathogenic fungi (Eroshin, Dedyukhina 2002; Fakas et al. 2009). *Mortierella* species are known as phosphate solubilizers

Table 2. Element concentration, C/N ratio, the content of humic acids, soil pH_{H2O} and soil moisture content in two depths in sampling plots. Data are means ± SD. *, significant (n = 3) differences between the soil samples from the two depths

Plot / Depth (cm)	Ca_{total} (g kg^{-1})	Mg_{total} (g kg^{-1})	K_{total} (g kg^{-1})	C_{total} (g kg^{-1})	P_{total} (g kg^{-1})	N_{total} (g kg^{-1})	C/N ratio	Humic acids (g kg^{-1})	pH_{H2O}	Soil moisture (%)
Pine 80										
0-10	1.32 ± 0.089*	0.56 ± 0.038*	0.44 ± 0.020*	155.8 ± 0.6*	0.11 ± 0.00*	3.9 ± 0.10*	40	70.9 ± 1.72*	4.02	31.29±3.03*
11-30	0.03 ± 0.002*	0.28 ± 0.019*	0.20 ± 0.009*	10.6 ± 0.1*	0.15 ± 0.01*	0.2 ± 0.00*	48	17.5 ± 0.09*	5.08	6.57±1.14*
Spruce 37										
0-10	1.81 ± 0.122*	0.50 ± 0.034*	0.33 ± 0.015*	247.0 ± 1.1*	0.16 ± 0.01*	6.7 ± 0.40*	37	95.2 ± 1.93*	4.01	47.83±2.07*
11-30	1.49 ± 0.101*	0.24 ± 0.016*	0.26 ± 0.012*	151.2 ± 4.0*	0.07 ± 0.00*	2.9 ± 0.40*	53	60.8 ± 1.43*	4.04	23.64±1.94*
Spruce 47										
0-10	4.54 ± 0.307*	1.08 ± 0.073*	0.80 ± 0.036*	439.4 ± 9.8*	0.29 ± 0.00*	13.3 ± 0.10*	33	96.4 ± 2.31*	4.01	52.40±4.58*
11-30	0.34 ± 0.023*	0.50 ± 0.034*	0.59 ± 0.027*	98.3 ± 3.0*	0.11 ± 0.01*	2.9 ± 0.00*	34	65.7 ± 0.03*	3.93	32.68±2.45*
Spruce 28										
0-10	2.26 ± 0.153*	0.89 ± 0.060*	0.70 ± 0.032*	434.1 ± 14.4*	0.31 ± 0.04*	14.0 ± 0.10*	48	135.4 ± 23.76*	3.92	71.68±5.48*
11-30	3.00 ± 0.203*	0.47 ± 0.032*	0.36 ± 0.016*	163.4 ± 1.8*	0.11 ± 0.01*	3.4 ± 0.20*	31	94.7 ± 0.68*	3.81	36.65±2.97*
Spruce 62										
0-10	3.32 ± 0.224*	0.83 ± 0.056*	0.50 ± 0.023*	427.7 ± 1.1*	0.28 ± 0.01*	13.8 ± 0.10*	31	89.3 ± 8.20*	3.94	60.00±3.85*
11-30	0.78 ± 0.053*	0.19 ± 0.013*	0.14 ± 0.006*	40.4 ± 0.3*	0.02 ± 0.00*	0.93 ± 0.10*	45	18. ± 2.94*	4.02	16.15±7.02*
Spruce 141										
0-10	2.22 ± 0.150*	0.62 ± 0.042*	0.48 ± 0.022*	301.5 ± 4.0*	0.21 ± 0.02*	10.8 ± 0.10*	28	106.2 ± 5.00*	4.22	62.07±3.86*
11-30	0.90 ± 0.061*	0.20 ± 0.014*	0.17 ± 0.008*	109.8 ± 6.3*	0.05 ± 0.01*	2.4 ± 0.30*	45	41.9 ± 2.19*	4.15	23.70±3.85*
Spruce 160										
0-10	0.58 ± 0.039*	0.72 ± 0.049*	0.58 ± 0.026*	291.8 ± 6.9*	0.18 ± 0.01*	8.1 ± 0.30*	36	87.9 ± 16.42*	3.72	53.25±4.50*
11-30	0.00 ± 0.000*	0.22 ± 0.015*	0.21 ± 0.010*	26.5 ± 0.9*	0.05 ± 0.01*	0.8 ± 0.10*	34	17.6 ± 1.07*	3.97	12.40±1.98*
Spruce 50										
0-10	3.47 ± 0.234*	0.96 ± 0.065*	0.65 ± 0.030*	459.5 ± 3.3*	0.29 ± 0.02*	17.2 ± 0.20*	27	86.4 ± 1.14*	4.18	67.60±4.10
11-30	2.17 ± 0.147*	0.73 ± 0.049*	0.54 ± 0.025*	310.8 ± 4.6*	0.19 ± 0.00*	10.0 ± 0.20*	31	126.5 ± 0.08*	3.95	62.76±4.33
Spruce 56										
0-10	3.34 ± 0.226*	1.06 ± 0.072*	0.73 ± 0.033*	510.4 ± 2.8*	0.33 ± 0.02*	16.8 ± 0.40*	30	105.6 ± 6.12*	4.03	56.19±4.39*
11-30	0.54 ± 0.036*	0.40 ± 0.027*	0.38 ± 0.017*	100.7 ± 1.1*	0.11 ± 0.00*	3.3 ± 0.20*	30	71.4 ± 0.75*	3.88	43.81±4.85*

and soil inoculation with propagules of these fungi has been shown to increase arbuscular mycorrhization of *Kostelelzkya virginica* in soil with increased salinity (Zhang et al. 2011).

Semihydromorphic soils also had significantly lower proportion of CFU of *Penicillium* spp. than in automorphic soils (13.95 ± 9.50 vs. 32.26 ± 12.12 %; $F = 1.64$, $p = 0.005$). In the soil of infected stands *Penicillium* spp. were less abundant than in healthy stands (12.09 ± 10.84 vs. 30.18 ± 12.51%; $F = 1.33$, $p = 0.02$). Semihydromorphic soils and soils from infected stands had practically no *Trichoderma* spp. (only the Spruce 56 plot had *Trichoderma* spp. 0.11% from all cultivable filamentous fungi). The incidence of root rot is associated with the lack of antagonistic fungi like *Trichoderma* and *Penicillum* spp. (Korhonen, Stenlid 1998), as also observed in our study. In our previous investigation where two infected spruce stands on authomorphic soils were analyzed, in the active vegetation period (from May to September), the relative number of *Penicillium* spp. was 33.20 to 38.39%, *Trichoderma* spp. 0.86 to 2.43%, and *Mortierella* spp. 9.26 to 13.63%, of all filamentous fungi in the soils (Grantina et al. 2012). In another investigation in Latvia where soil microbiology of twenty six 25- to 45-year-old spruce stands (forest types *Myrtillosa mel., Hylocomiosa, Oxalidosa*; soil types were not identified) were analyzed, soils of infected stands had lower abundance of *Trichoderma* spp., higher abundance of *Penicillium* spp., presence of several isolates of *Mortierella* also in soils of infected stands, and *Penicillium* showed antagonistic abilities against isolates of *H. annosum* S and P group in vitro (Arhipova et al. 2008).

Forest soil in the oldest stand Spruce 160 at depth of 11 to 30 cm had the highest number of represented fungal genera (10): *Aspergillus, Botrytis, Cladosporium, Humicola, Mortierella, Mucor, Penicillium, Trichoderma, Umbelopsis, Verticillium* and sterile mycelia. In other sampling plots, three to five genera and sterile mycelia were represented.

Total soil DNA

The amount of DNA isolated by a PowerSoil™ DNA

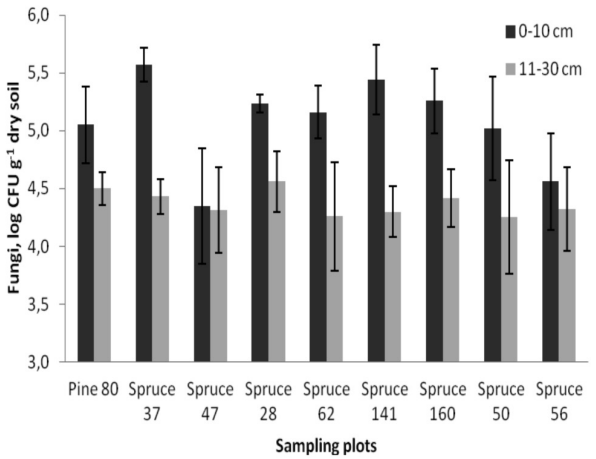

Fig. 1. Number of filamentous fungi in the studied soils (± SD; n = 9).

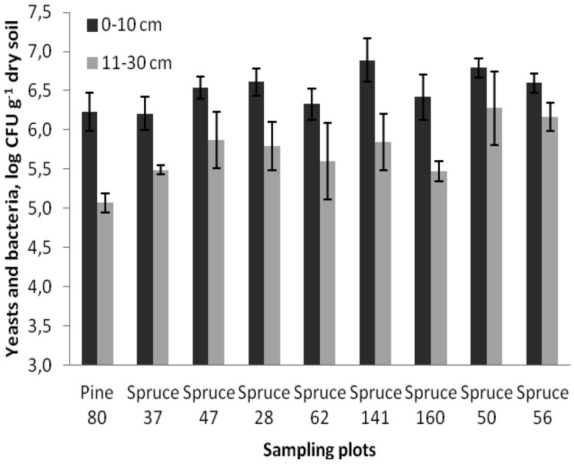

Fig. 2. Number of yeasts and maltose-utilizing bacteria in the studied soils (± SD; n = 9).

Isolation Kit ranged from 10.65 μg g^{-1} in deeper soil layers to 119.46 μg g^{-1} of dry soil in the upper soil horizon (Fig. 3). There were no significant differences between automorphic and semihydromorphic soils or between pine and spruce stands in the amount of total soil DNA but in all sampling plots the upper soil layer had significantly higher amounts of total soil DNA, as found elsewhere (Agnelli et al. 2004).

Fungal diversity

The estimated Shannon-Weaver diversity indices of *Ascomycota, Basidiomycota* and *Zygomycota* fungi using ARDRA are given in Fig. 4. There were no significant differences between pine and spruce stands in fungal diversity but the average fungal diversity *H'* in automorphic soils was significantly higher than in semihydromorphic soils, including infected stands (2.73 ± 0.33 vs. 2.18 ± 0.51; $F = 1.39$, $p = 0.009$). There were no significant differences in diversity between soil depth, with the exception of sampling plot Spruce 50 which had higher fungal diversity indices in the deepest soil layer. Similarly, it was found that in loblolly pine (*Pinus taeda*) forest mycorrhizal species predominated deeper in the soil profile while saprophytic species were more common in the litter layer (O' Brien et al. 2005).

Taxonomic analysis of soil fungal populations

A list of fungal genera determined according to morphological characters or DNA sequencing is given in Appendix 2. Of 59 sequenced isolates (28 species), 69.49% were *Ascomycota* (representing 17 genera), 28.81% were *Zygomycota* (three genera) and only 1.69% were *Basidiomycota* (one species *Thanatephorus cucumeris*). The low proportion of *Basidiomycota* is likely due to microbiological medium used (MEA) for isolation. In other investigations, it is known that *Basidiomycota* fungi dominate among all soil fungi in forests (Buee et al. 2009). In our previous investigations of forest soils using MEA, we isolated a few basidiomycetes like *Fomitopsis pinicola, Gloeophyllum sepiarium* (Grantina et al. 2010), and *Trichosporon porosum* (Grantina et al. 2012; Grantina-Ievina et al. in press), which represented only 1.19 to 1.36% of the total number of isolated and sequenced fungi. The best methods for studying soil basidiomycetes are hyphal isolation (Warcup 1951), collection of fruit-bodies and analysis of soil DNA (Luis et al. 2004).

The most abundant genus in all sampling plots and at both sampling depths was *Penicillium*. The following species were identified: *Penicillium canescens, Penicillium citreonigrum, Penicillium glaucoalbidum, Penicillium janthinellum, P. spinulosum* and *Penicillium thomii*. Both *P. citreonigrum* and *P. canescens* have been described in the literature as soil saprotrophic microfungi isolated from oak (*Quercus petraea*) forest with an acidic Cambisol in Czech Republic (Baldrian et al. 2011). *P. janthinellum* has been isolated from soil of an European aspen (*Populus tremula* L.) stand in Latvia (Grantina-Ievina et al. 2012). *P. spinulosum* has been isolated from an illuvial humus podzol soil of a spruce stand infected with *Heterobasidion parviporum* (Grantina et al. 2012). *P. canescens* was isolated from the soil of European aspen and hybrid aspen stands (*Populus tremuloides* Michx. × *Populus tremula* L.) (Grantina-Ievina et al. 2012) and from organic agriculture fields (Grantina et al. 2011). *P. glaucoalbidum,* which was isolated from the sampling plot Spruce 37, is known to be decomposer of spruce needle litter (Koukol et al. 2008).

The next most common genus isolated from all sampling plots except Spruce 50 was *Trichoderma* / *Hypocrea*. *Trichoderma asperellum, Trichoderma viride, Hypocrea pachybasioides* and *Hypocrea viridescens* (teleomorph of *Trichoderma viridescens*) have been isolated from spruce stands infected with *H. parviporum* (Grantina et al. 2012) and latter two species have been detected also in soil of aspen stands (Grantina-Ievina et al. 2012). *Hypocrea citrina* (teleomorph of *Trichoderma lacteum*) from the sampling

Table 3. Relative CFU of main fungal genera and sterile mycelia in two soil depths. Other genera: *Verticillium, Humicola* and *Cladosporium* spp.

Plot	Depth (cm)	*Mortierella* spp. (%)	*Penicillium* spp. (%)	*Trichoderma* spp. (%)	*Mucor* spp. (%)	*Aspergillus* spp. (%)	*Botrytis* spp. (%)	*Umbelopsis* spp. (%)	Other genera (%)	Sterile mycelia (%)
Pine 80	0-10	13.19	38.57	15.20	0.07	0.00	0.00	0.00	6.59	26.37
	11-30	0.16	31.80	1.82	0.00	0.78	0.00	0.00	0.78	64.67
Spruce 37	0-10	30.45	31.12	10.45	7.48	0.00	0.00	9.80	0.00	10.71
	11-30	21.07	30.77	16.87	7.75	0.00	0.00	1.76	0.00	21.78
Spruce 47	0-10	0.00	22.63	1.06	0.11	0.00	0.00	41.44	0.00	34.76
	11-30	0.00	12.23	1.95	0.00	0.00	0.00	22.08	0.00	63.73
Spruce 28	0-10	12.07	36.20	37.19	0.63	0.00	0.00	0.49	0.00	13.41
	11-30	46.11	14.85	7.76	1.23	0.00	0.00	0.18	0.00	29.88
Spruce 62	0-10	14.26	47.00	7.23	1.10	0.00	0.00	8.08	0.00	22.34
	11-30	14.53	15.68	4.81	0.03	0.00	0.00	0.11	0.00	64.83
Spruce 141	0-10	3.71	41.89	29.18	3.14	0.00	0.28	4.81	0.00	16.98
	11-30	53.30	19.19	6.61	0.03	0.00	0.00	1.69	0.00	19.19
Spruce 160	0-10	19.38	53.76	4.02	4.18	0.00	0.00	7.31	0.00	11.34
	11-30	1.08	26.31	5.17	0.16	10,38	5.64	1.41	25.37	24.48
Spruce 50	0-10	0.00	10.06	0.00	0.92	0.00	0.00	83.49	0.00	5.53
	11-30	0.00	10.80	0.00	0.03	0.00	0.00	50.20	0.00	38.97
Spruce 56	0-10	3.72	26.80	0.11	0.00	0.00	0.00	42.96	0.00	26.41
	11-30	0.31	0.65	3.12	0.00	0.00	0.00	3.77	0.00	92.14

plot Spruce 56 was isolated also from leaf litter and rich soils in Europe, Japan and North America (Overton et al. 2006).

Several isolates from the genus *Lecythophora* (*Lecythophora mutabilis* and *Lecythophora* sp.) were isolated from bulk soil in sampling plots Pine 80, Pruce 62 and Spruce 56. *L. mutabilis* has been detected in the rhizosphere of prairie grass *Andropogon gerardii* (Jumponen 2011), isolated as a root endophyte of *Betula papyrifera* and *Abies balsamea* in boreal forests in eastern Canada (Kernaghan, Patriquin 2011), and from roots of *Pinus sylvestris* seedlings (Menkis, Vasaitis 2011). *L. mutabilis* has been isolated from the illuvial humus podzol soil of spruce stand infected with *Heterobasidion parviporum* (Grantina et al. 2012).

The entomopathogenic species *Beauveria bassiana* was isolated from two sampling plots Spruce 37 and Spruce 141. This species has been isolated from sod-podzolic soil of a spruce stand infected with *H. parviporum* (Grantina et al. 2012). In other stduies this species has been isolated from roots of healthy pine *Pinus sylvestris* trees but not from trees in decline (Lygis et al. 2004). Another entomopathogenic species *Tolypocladium cylindrosporum* (synonym *Beauveria*

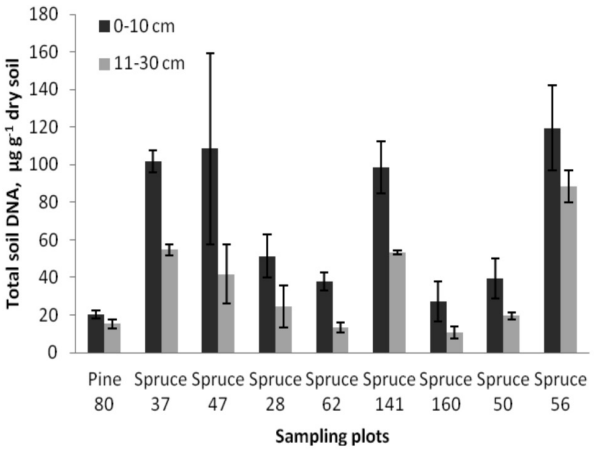

Fig. 3. The amount of total soil DNA in sampling plots at two depths (± SD; n = 3).

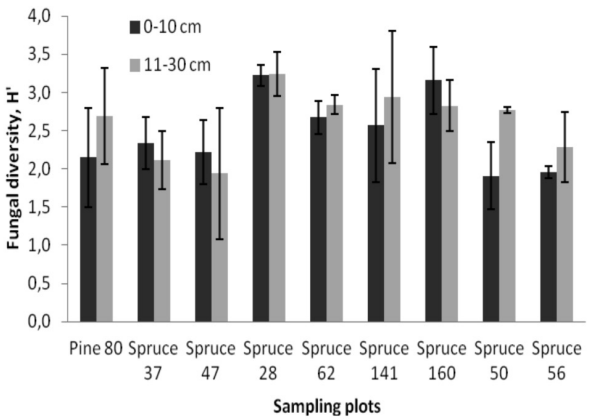

Fig. 4. The average fungal diversity *H'* at two soil depths in sampling plots (± SD; n = 3).

cylindrospora) was isolated from soil of Spruce 28 and Spruce 160. In a study conducted in Šumava National Park in Czech Republic this species was isolated from soil under spruce colonized with *Ips typographus* (Landa et al. 2001).

Pochonia bulbillosa was isolated from three sampling plots, Spruce 37, Spruce 62 and Spruce 141. Previously this species has been isolated from illuvial humus podzol soil of a spruce stand infected with *H. parviporum* (Grantina et al. 2012), from peatlands (Thormann et al. 2004) and as a decomposer of spruce wood (Thormann 2006).

Stilbella species were isolated from three sampling plots, Spruce 37, Spruce 141 and Spruce 160. *Stilbella byssiseda* (Spruce 141) has been detected in soil of *Salix herbacea* in the Austrian Central Alps (Oberkofler, Peintner 2008). This fungus is known to colonize the fructifications of myxomycetes (Rogerson, Stephenson 1993).

The highest fungal diversity in the terms of the number of represented genera (16 genera) was in the oldest sampling plot Spruce 160. Two *Mucorales* were identified as *Absidia psychrophilia* and *Ambomucor seriatoinflatus,* and four isolates with sterile mycelia as *Chalara longipes, Thanatephorus cucumeris, Stilbella* sp. and *Tolypocladium cylindrosporum*. In other sampling plots, the number of genera ranged from three to 10. The lowest diversity (only three to six genera) was in semihydromorphic soils (Spruce 47, Spruce 50, Spruce 56), of which two were infected with *H. annosum s.l.* (Spruce 50, Spruce 56). This is consistent with the obtained ARDRA results.

Results of stepwise multiple regression analysis

Results of stepwise multiple regression analysis are summarized in Table 4. Total amount of soil DNA and fungal diversity estimated as the Shannon-Weaver diversity index (*H'*) were not significantly affected by any of the factors included in the regression models (not listed in Table 4). Multiple regression analysis showed that elevated concentrations of magnesium negatively affected the number of filamentous fungi and number of represented fungal genera. Elevated concentration of potassium positively affected the number of yeasts and maltose-utilizing bacteria, but negatively affected the proportion of *Mortierella* spp.

The genera *Penicillium* and *Mortierella* were chosen for regression analysis as they were abundant in all sampling plots. Forest age had significant impact on proportion of *Penicillium* spp. CFU, especially in the upper soil layer 0 to 10 cm ($r = 0.63$, $R^2 = 0.40$; Fig. 5A), and total number of fungal genera, particularly in the deepest soil layer 11 to 30 cm ($r = 0.77$, $R^2 = 0.60$; Fig. 5B). Positive impact of forest age has previously been observed on the abundance of mat-forming fungi in soil and total mat-forming taxon abundance in a mountain hemlock ecotype (Trappe et al. 2012).

Antagonism assay

Results of the antagonism assay are summarized in Appendix 1. Examples are given in Fig. 6. According to the growth in control Petri dishes, after seven days of incubation the *Penicillium* isolates were divided into three groups according to significant differences ($p < 0.05$) in growth kinetics: slow-growing with average growth rate 0.03 to 0.06 mm h^{-1}, medium fast-growing isolates with average growth rate 0.07 to 0.10 mm h^{-1} and fast-growing isolates with average growth rate 0.11 to 0.14 mm h^{-1}. The average growth rate of root rot isolates in the control plates was 0.23 to 0.30 mm h^{-1}. In particular cases the growth rate of *Penicillium* isolates changed significantly on Petri dishes in the antagonism assay (Table 5). The average growth rate of slow- and medium fast-growing isolates was significantly higher in several cases, but the average growth

Table 4. Multiple regression analysis (numbers in the columns are p values). MEA, malt extract agar. NS, not significant; –, negative impact

Factor	Yeasts and maltose-utilizing bacteria on MEA	Filamentous fungi on MEA	Percentage of *Penicillium* spp.	Percentage of *Mortierella* spp.	Total number of fungal genera
Soil sampling depth	0.0007 –	< 0.001 –	0.0007 –	NS	NS
Soil moisture content	< 0.0001	NS	0.03 –	NS	0.04 –
Soil pH_{H2O}	NS	NS	NS	NS	0.008 –
Forest age	NS	NS	0.003	NS	0.0008
Total calcium content	NS	NS	NS	NS	NS
Total magnesium content	NS	< 0.0002 –	NS	NS	0.006 –
Total potassium content	0.046	NS	NS	0.01 –	NS
C/N ratio	0.04a	NS	0.01	NS	NS
Total phosphorus content	0.05a	0.04	NS	NS	0.005
Humic acids	NS	0.004	0.04	NS	NS
Multiple R^2	0.95	0.98	0.80	0.35	0.75
p-value of the final model	< 0.0001	< 0.0001	0.0006	0.04	0.002

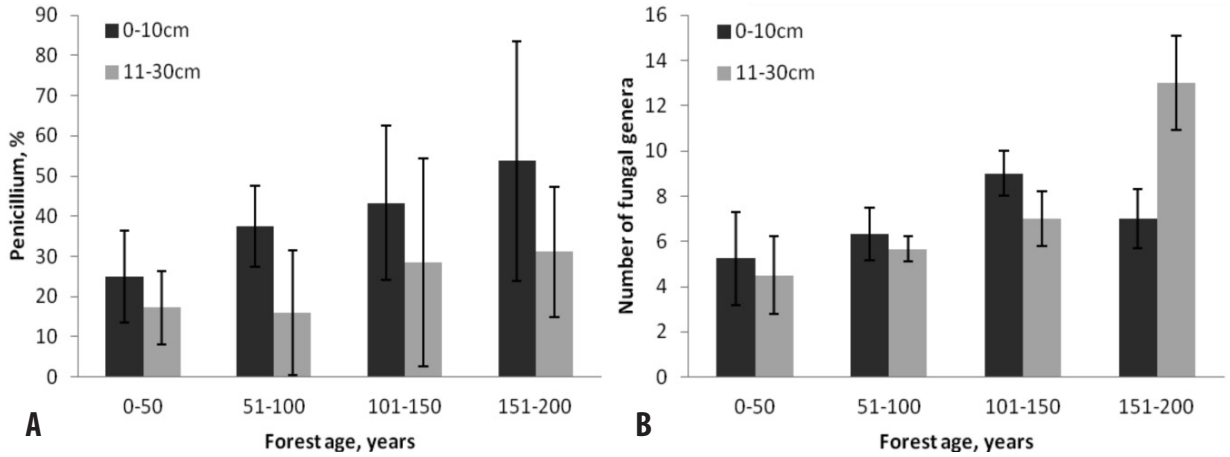

Fig. 5. Relative abundance of *Penicillium* spp. (A) and number of fungal genera depending on forest age (B).

rate of the fast-growing group was significantly reduced in antagonism pairings with all root rot isolates. The average growth rate of root rot isolates was lower by more than twofold (by 53 to 57%).

In 62% of all pairings an antagonistic zone was observed where both fungal hyphae came into contact but neither of the fungi overgrew each other. In 21% of pairings the inhibition zone after one month of incubation was still present. In the case of *P. citreonigrum,* a 1.50 to 3.75 mm wide inhibition zone was observed in pairings with all root

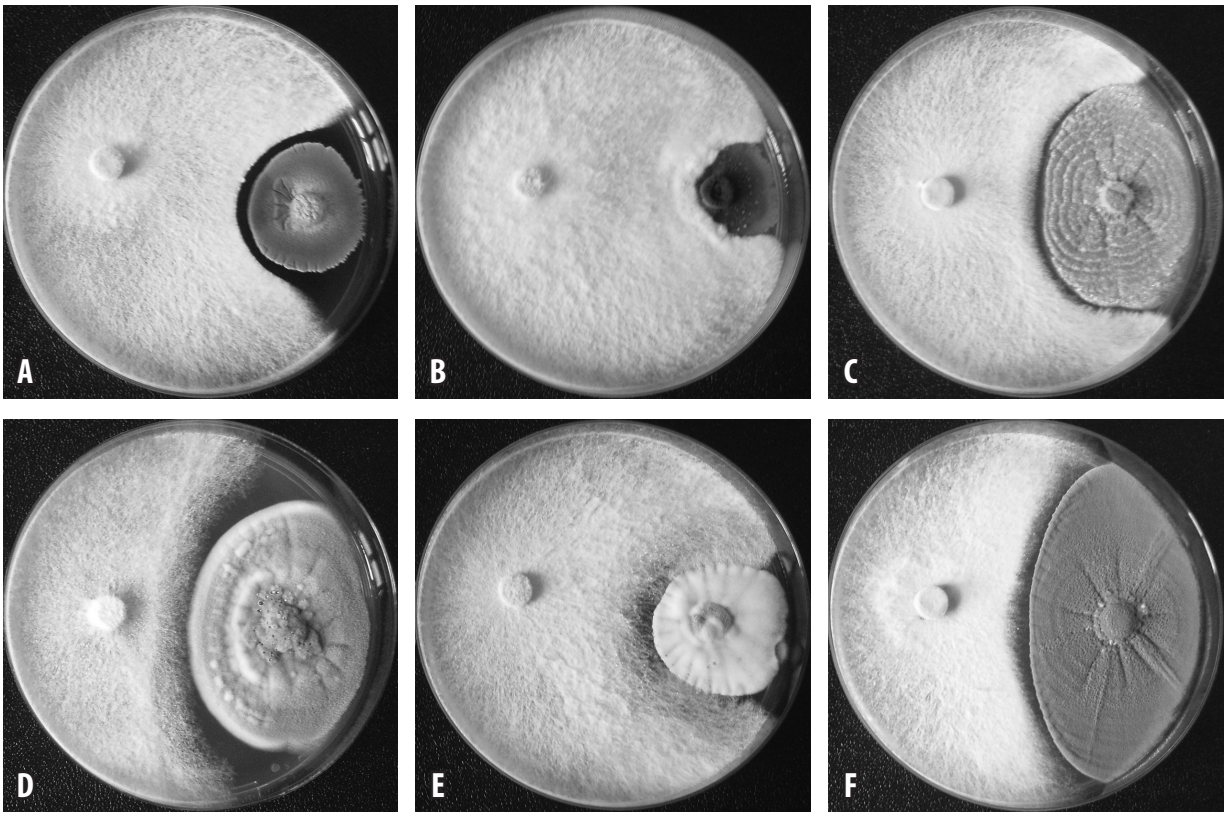

Fig. 6. Examples of antagonism assay: inhibition zone is present after one month of incubation, MSCL 981 and *P. verruculosum* MSCL 882 (A); zone of antagonism is not present and isolate of root rot overgrows *Penicillium* sp., MSCL 980 and *P. montanense* MSCL 942 (B); zone of antagonism is present but isolate of root rot partially overgrows *Penicillium* sp., S 56 and *Penicillium* sp. MSCL 1153 (C); zone of antagonism is present but fungal hyphae come into contact although none of the fungi overgrows other, MSCL 1021 and *P. canescens* MSCL 1213 (D); both fungi overgrow each other in the zone of interaction, S 56 and *P. roseopurpureum* MSCL 1220 (E); *Penicillium* sp. overgrows the isolate of root rot in the antagonism zone, S 56 and *P. thomii* MSCL 1148 (F).

Table 5. Relative changes in the average growth rate of *Penicillium* spp. isolates in the presence of different root rot isolates on Petri dishes in antagonism assay, in comparison to the control agar plates. *, significant differences among *Penicillium* groups, *p* values indicate significant changes in growth speed in comparison with control agar plates. MSCL, Microbial Strain Collection of Latvia

Group of *Penicillium*	MSCL 980	MSCL 981	MSCL 1020	MSCL 1021	S 56
Slow growing	120.69 ± 29.64 %* ($p = 0.003$)	138.32 ± 30.77 %* ($p < 0.0001$)	138.32 ± 30.77 %* ($p < 0.0003$)	102.12 ± 16.28 %	114.57 ± 12.72 % ($p = 0.0009$)
Medium fast growing	102.67 ± 17.50 %*	112.83 ± 22.43 %* ($p = 0.02$)	111.13 ± 16.92 % ($p < 0.01$)	97.09 ± 10.29 %	103.54 ± 22.25 % ($p = 0.005$)
Fast growing	71.88 ± 22.90 %* ($p = 0.001$)	76.09 ± 29.16 %* ($p = 0.008$)	73.32 ± 28.36 %* ($p < 0.01$)	80.95 ± 9.78 %* ($p = 0.0001$)	72.46 ± 16.03 %* ($p = 0.02$)

rot isolates. Only two isolates (0.8%) overgrew the isolate of root rot in the antagonism zone, *P. swiecickii* in the case of MSCL 1021 and isolate *P. thomii* in the case of S56. In 4.4% of all pairings both fungi overgrew each other in the zone of interaction. In other cases (11.8%), isolates of the root rot fungi fully or partially overgrew the *Penicillium* sp. The efficiency of *Penicillium* in suppressing radial growth of isolates of root rot fungi ranged from 26 to 84% (average 43 to 57% for aparticular isolate of root rot fungus). *Penicillium* isolates with the highest efficiency were medium- and fast-growing. *P. griseofulvum* var. *dipodomyicola* MSCL 874, isolated from the agricultural soil, had efficiency 59 to 82%, *P. brasilianum* MSCL 1229 had efficiency 46 to 69% and *P. canescens* MSCL 1213 – 54 to 69%. Both of the latter were isolated from soil of aspen stands. Efficiency of 83 to 84% in suppressing radial growth of the pathogen was observed in the case of two *Penicillium* isolates from aspen stands: *P. canescens* MSCL 1091 with *H. annosum s.s.* MSCL 1021, *Penicillium* sp. O48 with *H. annosum s.l.* S56 and one isolate from an infected spruce stand *P. spinulosum* MSCL 1145 with *H. parviporum* MSCL 980.

In general, the results obtained for isolates of the same species and *Penicillium* isolates from agricultural soil, former agricultural soil planted with aspen stands and forest soil showed similar results. *Penicillium* spp. in general reduced the growth rate of *H. annosum s.l.* and some isolates produced some antifungal components, as described previously (Yang et al. 2008) but the antagonistic effect was not as strong as in the case of *Trichoderma* spp. (Nikolajeva et al. 2012).

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Appendix 1. List of *Penicillium* isolates used in antagonism assay, their growth rate on 3% MEA and antagonistic abilities against root rot isolates MSCL 980, MSCL 981, MSCL 1020, MSCL 1021 and S56. If the inhibition zone after one month of incubation was still present its width is indicated in millimeters. (1), soil of European aspen (*Populus tremula*) and hybrid aspen (*Populus tremuloides* × *P. tremula*) stands (Grantina-Ievina et al. 2012); (2), soil of infected spruce (*Picea abies*) stands (Grantina et al. 2012.); (3), the present study; (4), agricultural soil (Grantina et al. 2011). 0, zone of antagonism is present but fungal hyphae come into contact although none of the fungi overgrows other; 00, both fungi overgrow each other in the zone of interaction; 0/–, zone of antagonism is present but isolate of root rot partially overgrows *Penicillium* sp.; +, *Penicillium* sp. overgrows the isolate of root rot in the antagonism zone; –, zone of antagonism is not present and isolate of root rot overgrows *Penicillium* sp. MSCL, Microbial Strain Collection of Latvia

Species (reference)	MSCL strain number	Growth (mm h^{-1})	MSCL 980	MSCL 981	MSCL 1020	MSCL 1021	S 56
Slow growing isolates							
P. canescens (1)	1119	0.03 ± 0.004	00	0	0	4.0	1.5
P. canescens (1)	1209	0.05 ± 0.002	2.5	0	0	6.0	3.5
P. montanense (2)	942	0.05 ± 0.000	–	0	0	0/–	0
P. canescens (1)	1227	0.06 ± 0.002	3.0	2.0	1.5	0	2.3
P. canescens (1)	1210	0.06 ± 0.000	0	0.5	0	–	0
P. canescens (1)	1208	0.06 ± 0.000	2.0	0.5	0	0/–	0
P. canescens (1)	1214	0.06 ± 0.002	0	0	0	0	0
P. canescens (1)	1109	0.06 ± 0.000	3.7	3.0	1.5	0	2.0
P. canescens (1)	1225	0.06 ± 0.003	2.0	0	0	–	0
P. canescens (1)	1211	0.06 ± 0.002	1.5	1.0	1.5	0	4.0
P. canescens (1)	1224	0.06 ± 0.005	1.5	0	0	–	1.5
P. canescens (1)	1215	0.06 ± 0.003	0	0	0	–	0
P. citreonigrum (3)	1150	0.06 ± 0.002	2.5	1.5	3.3	3.8	3.3
P. montanense (1)	1228	0.06 ± 0.002	3.5	1.0	0	–	0
P. roseopurpureum (1)	1220	0.06 ± 0.002	0	0	0	–	00
P. roseopurpureum (1)	1218	0.06 ± 0.000	0	0	0	–	00
P. roseopurpureum (1)	1219	0.06 ± 0.002	0	0	0	–	00
Penicillium sp. (3)	1152	0.06 ± 0.002	–	0	0	0/–	0
Penicillium sp. (3)	1137	0.06 ± 0.001	–	0	0	–	0
P. verruculosum (4)	882	0.06 ± 0.008	0	1.5	0	–	0
Medium fast growing isolates							
P. canescens(4)	879	0.07 ± 0.008	2.3	0	0	0/–	2.6
P. canescens (1)	1223	0.07 ± 0.001	1.5	0	0	0	0
P. canescens (1)	1104	0.07 ± 0.000	1.5	0	0	3.3	0
P. canescens (1)	1110	0.07 ± 0.006	0	0	0	1.5	1.0
P. canescens (1)	1213	0.07 ± 0.001	0	2.5	0	0	0
P. canescens (1)	1216	0.07 ± 0.002	0	0	0	–	0.8
P. canescens (1)	1107	0.07 ± 0.003	0	0	1.5	–	0
P. canescens (1)	-	0.07 ± 0.002	0	0	0	0	0
P. corylophilum (1)	1135	0.07 ± 0.001	–	0	0	–	0
Penicillium sp. (1)	-	0.07 ± 0.000	0	0	0	2.8	0
P. spinulosum (3)	1138	0.07 ± 0.001	–	0	-	–	-
P. swiecickii (1)	1221	0.07 ± 0.000	0	0	0	0	0
P. aurantiogriseum (4)	876	0.08 ± 0.002	0	0	0	0/–	0
P. aurantiogriseum (1)	-	0.08 ± 0.002	1.5	0	0	3.5	2.0
P. canescens (1)	875	0.08 ± 0.009	0	0	0	–	0
P. canescens (1)	1212	0.08 ± 0.002	0	1.8	0	1.0	0
P. canescens (1)	1226	0.08 ± 0.001	0	1.5	0	4.0	0
P. canescens (1)	-	0.08 ± 0.002	1.5	0	0	0	0
P. swiecickii (1)	1217	0.08 ± 0.002	0	0	0	+	0
P. canescens (1)	1091	0.10 ± 0.000	0	0	0	1.0	0
P. swiecickii (1)	1222	0.10 ± 0.072	1.5	0	0	0	0
Fast growing isolates							
P. canescens (1)	1093	0.11 ± 0.004	0	0	1.5	0/–	0
P. melanoconidium (4)	871	0.11 ± 0.080	0	0	0	–	0
Penicillium sp. (3)	1153	0.11 ± 0.076	00	0	0	–	0/-
P. thomii (3)	1148	0.12 ± 0.002	00	0	0	–	+
P. brasilianum (1)	1229	0.13 ± 0.004	0	0	0	–	0

Appendix 2. *continued*

Species (reference)	MSCL strain number	Growth (mm h^{-1})	MSCL 980	MSCL 981	MSCL 1020	MSCL 1021	S 56
P. griseofulvum var. *dipodomyicola* (4)	874	0.13 ± 0.000	0	0	0	0/–	0
P. janthinellum (1)	1097	0.13 ± 0.001	0	0	0	00	0
P. canescens (1)	1095	0.14 ± 0.001	0.5	0	0	–	0
P. commune (4)	878	0.14 ± 0.002	00	0	0	–	0
P. spinulosum (3)	1145	0.14 ± 0.001	00	0	0	00	0
P. spinulosum (2)	952	0.14 ± 0.001	00	0	0	–	0

Appendix 2. List of fungal species determined in the soil of sampling plots according to morphological characters or ribosomal DNA sequencing results (indicated with the number of homologue sequence at NCBI data base). MSCL, Microbial Strain Collection of Latvia

Sampling plot; depth (cm)	Species	Homologue sequence, NCBI acc. No.	Maximum identity (%)	E value	Strain No. in MSCL
Pine 80; 0–10	*Humicola* sp.	–	–	–	–
	Lecythophora mutabilis	HM036599.1	100	0	–
	Lecythophora sp.	AY219880.1	95	0	–
	Mortierella sp.	HQ022201.1	99	0	–
	Mucor sp.	–	–	–	
	Penicillium citreonigrum	AY157489.1	96	0	1150
	Phoma herbarum	AF218792.1	98	0	–
	Trichoderma sp.	–	–	–	–
Pine 80; 11–30	*Aspergillus cervinus*	–	–	–	–
	Cladosporium sp.	–	–	–	–
	Mortierella sp.	FJ810149.1	100	0	–
	Mortierella sp.	FJ810149.1	100	0	–
	Penicillium sp.	–	–	–	–
	Sagenomella sp.	GQ169325.1	98	0	–
	Trichoderma sp.	–	–	–	–
Spruce 37; 0–10	*Beauveria bassiana*	GU566276.1	99	0	1055
	Hypocrea pachybasioides	GU934589.1	99	0	1072
	Mortierella sp.	HQ022201.1	99	0	–
	Mucor sp.	–	–	–	–
	Penicillium sp.	–	–	–	–
	Pochonia bulbillosa	AB378552.1	99	0	1056
	Trichoderma sp.	–	–	–	–
	Umbelopsis sp.	–	–	–	–
Spruce 37; 11–30	*Hypocrea pachybasioides*	AY240841.1	98	0	–
	Mortierella sp.	–	–	–	–
	Mucor sp.	–	–	–	–
	Stilbella sp.	HQ631053.1	99	0	1057
	Penicillium glaucoalbidum	FJ903357.1	95	0	–
	Umbelopsis sp.	–	–	–	–
Spruce 47; 0–10	*Mucor* sp.	–	–	–	–
	Penicillium sp.	–	–	–	–
	Trichoderma asperellum	FJ908743.1	98	0	–
	Umbelopsis sp.	–	–	–	–
Spruce 47; 11–30	*Hypocrea viridescens*	GU566274.1	99	0	–
	Penicillium janthinellum	GU212865.1	98	0	1137
	Penicillium thomii	GU934556.1	100	0	1148
	Umbelopsis sp.	–	–	–	–

Appendix 2. *continued*

Sampling plot; depth (cm)	Species	Homologue sequence, NCBI acc. No.	Maximum identity (%)	E value	Strain No. in MSCL
Spruce 28; 0–10	*Mortierella macrocystis*	AJ878782.1	96	0	–
	Mucor sp.	–	–	–	–
	Paecilomyces carneus	AB258369.1	100	0	1132
	Penicillium spinulosum	GU566252.1	100	0	1145
	Tolypocladium cylindrosporum	AB208110.1	97	0	1129
	Trichoderma viride	FJ872073.1	99	0	–
	Umbelopsis sp.	–	–	–	–
Spruce 28; 11–30	*Ambomucor seriatoinflatus*	AY743664.1	80	1,00E–61	1142
	Mortierella macrocystis	AJ878782.1	96	0	–
	Penicillium sp.	–	–	–	–
	Trichoderma sp.	–	–	–	–
	Umbelopsis sp.	–	–	–	–
	Zalerion varium	AJ608987.1	100	0	1102
Spruce 62; 0–10	*Absidia psychrophilia*	AY944874.1	93	0	1139
	Lophiostoma cynaroidis	EU552138.1	90	5,00E–145	–
	Mortierella macrocystis	AJ878782.1	99	0	–
	Mortierella macrocystis	AJ878782.1	96	0	–
	Penicillium sp.	–	–	–	–
	Pochonia bulbillosa	AB378551.1	100	0	–
	Trichoderma sp.	–	–	–	–
	Umbelopsis sp.	–	–	–	–
	Zygomycete	AM292200.1	96	0	–
Spruce 62; 11–30	*Clonostachys candelabrum*	AF210668.1	98	0	1054
	Hypocrea pachybasioides	GU934589.1	99	0	–
	Lecythophora mutabilis	HM036599.1	99	0	–
	Mortierella sp.	–	–	–	–
	Mucor sp.	–	–	–	–
	Umbelopsis sp.	–	–	–	–
Spruce 141; 0–10	*Beauveria bassiana*	AJ560684.1	98	0	1094
	Botrytis sp.	–	–	–	–
	Mortierella sp.	–	–	–	–
	Mucor sp.	–	–	–	–
	Oidiodendron sp.	HM208724.1	98	0	1125
	Penicillium sp.	–	–	–	–
	Stilbella byssiseda	AF335453.1	93	0	–
	Trichoderma sp.	–	–	–	–
	Umbelopsis sp.	–	–	–	–
Spruce 141; 11–30	*Beauveria bassiana*	AY532056.1	99	0	1100
	Mortierella sp.	–	–	–	–
	Mucor sp.	–	–	–	–
	Penicillium sp.	–	–	–	–
	Pochonia bulbillosa	AB378554.1	95	0	–
	Trichoderma sp.	–	–	–	–
	Umbelopsis sp.	–	–	–	–
Spruce 160; 0–10	*Chalara longipes*	FR717230.1	98	0	–
	Hypocrea pachybasioides	AY240841.1	100	0	1115
	Mortierella sp.	EU240133.1	99	0	–
	Mucor sp.	–	–	–	–
	Penicillium sp.	–	–	–	1153
	Thanatephorus cucumeris	EF155506.1	99	0	–
	Umbelopsis sp.	–	–	–	–

Appendix 2. *continued*

Sampling plot; depth (cm)	Species	Homologue sequence, NCBI acc. No.	Maximum identity (%)	E value	Strain No. in MSCL
Spruce 160; 11–30	*Absidia psychrophilia*	AY944874.1	94	0	–
	Ambomucor seriatoinflatus	AY743664.1	99	0	1092
	Aspergillus cervinus	–	–	–	–
	Botrytis sp.	–	–	–	–
	Cladosporium sp.	–	–	–	–
	Humicola sp.	–	–	–	–
	Mortierella macrocystis	AJ878782.1	99	0	1136
	Mortierella sp.	FJ810149.1	99	0	1053
	Mortierella sp.	FJ810149.1	99	0	–
	Penicillium canescens	FJ439586.1	99	0	–
	Penicillium spinulosum	GU566247.1	98	0	1138
	Stilbella sp.	HQ631053.1	99	0	1144
	Tolypocladium cylindrosporum	AB208110.1	97	0	–
	Trichoderma sp.	–	–	–	–
	Umbelopsis sp.	–	–	–	–
	Verticillium sp.	–	–	–	–
Spruce 50; 0–10	*Penicillium* sp.	–	–	–	–
	Mucor sp.	–	–	–	–
	Umbelopsis sp.	–	–	–	–
Spruce 50; 11–30	*Penicillium* sp.	–	–	–	–
	Mucor sp.	–	–	–	–
	Umbelopsis sp.	–	–	–	–
Spruce 56; 0–10	*Mortierella* sp.	–	–	–	–
	Penicillium sp.	GU446648.1	97	0	1152
	Talaromyces sp.	GU827480.1	99	0	1147
	Trichoderma sp.	–	–	–	–
	Umbelopsis sp.	–	–	–	–
Spruce 56; 11–30	*Mortierella* sp.	–	–	–	–
	Hypocrea citrina	DQ000622.1	99	0	–
	Lecythophora mutabilis	HM036599.1	99	0	–
	Penicillium sp.	–	–	–	–
	Umbelopsis sp.	–	–	–	–

3.5. Comparison of soil microorganism abundance and diversity in the stands of European aspen (*Populus tremula* L.) and hybrid aspen (*Populus tremuloides* Michx. × *P. tremula* L.).

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Comparison of soil microorganism abundance and diversity in stands of European aspen (*Populus tremula* L.) and hybrid aspen (*Populus tremuloide*s Michx. × *P. tremula* L.)

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Abstract. The use of short rotation forest tree species is increasing worldwide. The hybrid aspen (*Populus tremuloide*s Michx. × *P. tremula* L.) is one of the suitable tree species under the climatic conditions of the Baltic region. The cultivation of these trees on former agricultural soils differs from agricultural practices with reduced soil tillage and is characterized by increased demand of nutrients, which in long term can cause changes in the soil microbial populations. The aim of our investigation was to compare soil microbial populations in hybrid aspen and European aspen (*P. tremula* L.) stands in four sampling plots with aspen age ranging from 10 to 46 years. The abundance and diversity of soil microbial populations were estimated by enumeration of microorganisms (plate counts on three microbiological media) and by molecular methods (PCR, ARDRA, molecular identification of fungal isolates). Results showed that during long cultivation periods hybrid aspens reduced the number of culturable bacteria. The number of culturable filamentous fungi was statistically significantly increased only in one sampling plot in soil samples from hybrid aspen clones at a depth of 16–30 cm and only on one microbiological cultivation medium. The same was detected also with molecular methods in the case of fungal diversity estimated by Shannon–Weaver diversity indices in this sampling plot. None of the other characteristics of soil microbial populations, such as the number of yeasts and maltose utilizing bacteria on MEA, the number of yeasts and filamentous fungi on RBA, the total amount of soil DNA, fungal and bacterial diversity estimated by molecular biology methods, and species composition of filamentous fungi, was significantly affected by hybrid aspen. The identified filamentous fungi represented the following genera: *Acremonium*, *Exophiala*, *Geomyces*, *Gibellulopsis*, *Gibberella*, *Hypocrea*/*Trichoderma*, *Leptosphaeria*, *Metarhizium*, *Mortierella*, *Nectria*, *Paecilomyces*, *Penicillium*, *Trichosporon*, and others. The main conclusion was that cultivation of hybrid aspen as a short rotation forest tree in the Baltic region would not significantly affect the abundance and diversity of saprophytic soil microorganisms.

Key words: hybrid aspen, soil microorganisms, microbial diversity, soil filamentous fungi.

INTRODUCTION

The use of short rotation forest tree species has been increasing worldwide since the 1970s (Stanton et al., 2002). One of the suitable tree species for the climatic conditions of the Baltic region is hybrid aspen (*Populus tremuloide*s Michx. × *P. tremula* L.).

Hybrid aspen has been traditionally cultivated on former agricultural lands (since 1999 in Estonia) (Tullus et al., 2007) or on contaminated soils, and since 2000 in oil shale opencasts in Estonia (Tullus et al., 2008). Nevertheless, taking into account the possible environmental impacts of monospecific plantations with one tree species, cultivation of hybrid aspen is not recommended on traditional forest lands in Estonia (Tullus, 2010). In Latvia the genetic selection of hybrid aspen began in the 1960s and new scientific plantations have been established in recent decades (Zeps et al., 2008). At present there are over 4500 ha of hybrid aspen plantations in Northern Europe (Tullus et al., 2012).

Fast growing tree species can be favourable for the environment by decreasing soil erosion and surface runoff due to their root system and vegetation in comparison to conventional agriculture. This positive effect is detectable already in three to five years even in eroded soils (Mann & Tolbert, 2000). The possible adverse effects to the environment in the context of soil are connected with increased demand of nutrients. It is estimated that in poplar plantations the maximum uptake of nutrients occurs at the age of 5–6 years (Nelson et al., 1987), but for aspen stands in boreal climate it is observed that at the age of 9 years a rapid increase in annual biomass production occurs. Because of high establishment costs, hybrid aspen plantations are usually established with low planting densities. Thus the initial productivity per hectare is rather low and maximum nutrient uptake occurs at an older age compared to poplars and natural aspen stands; for example, the nutrient content in the above-ground biomass of 7-year-old hybrid aspen plantations could be only 0.5–3.5% of the total soil nutrient pool (Tullus et al., 2009). In addition, the growth of fast growing tree clones (*Salix* and *Populus* spp.) is characterized by increased litter amounts and an elevated C/N ratio and lignin content in the litter (Baum et al., 2009).

Cultivation of short rotation energy crops on former agricultural soils differs from agricultural practices with reduced soil tillage, which in long term can cause changes in the vertical distribution of soil microorganisms: an increased microbial biomass in the upper 5 cm of soil and a decreased biomass in subsoil (Makeschin, 1991). Cultivation of poplars and willows introduces ectomycorrhizal fungi in the former agricultural soils that normally contain saprophytic fungi and increases the diversity of basidiomycetes in general (Lynch & Thorn, 2006). In Germany Baum & Hrynkiewicz (2006) in their investigation of the rhizosphere and bulk soil of two willow clones (*Salix viminalis* and *S.* × *dasyclados*) differing in their mycorrhizal colonization and the decomposition rates of their litter found the species composition of saprophytic fungi to be tree clone specific. The lower mycorrhizal colonization and higher litter decomposition rate of *S. viminalis* brought about a higher number of saprophytic fungal species. The authors found

that colonization densities of seven saprophytic species (*Acremonium butyri*, *Cladosporium herbarum*, *Cylindrocarpon destructans*, *Penicillium janthinellum*, *Penicillium spinulosum*, *Plectosphaerella cucumerina*, and *Trichoderma polysporum*) significantly correlated with the acid-phosphatase and arylsulphatase activity in the rhizosphere. It is known that also *Populus* species and clones can differ in their mycorrhizal colonization rate and species composition as it was detected in the investigation of *Populus trichocarpa* and *P. tremula* L. × *P. tremuloide*s Michx. (Baum & Makeschin, 2000) and that hybrid aspen (*P. tremuloide*s Michx. × *P. tremula* L.) has an increased root biomass in comparison to European aspen (Nikula et al., 2009). Other investigations exist about the mycorrhizal colonization of *Populus* spp. (Cripps & Miller, 1995; Kaldorf et al., 2004) but there is a lack of information regarding the species composition of saprophytic fungi and bacteria in the soil of these trees. In the review article of Tullus et al. (2012) the authors conclude that there is a need for deeper investigations considering the environmental and biodiversity impact of hybrid aspen plantations.

The aim of our investigation was to compare soil microbial populations, mainly saprophytic fungi and bacteria, in hybrid aspen (*P. tremuloide*s Michx. × *P. tremula* L.) and European aspen (*P. tremula* L.) plantations on former agricultural lands. The hypothesis was that hybrid aspen plantations would cause changes in soil microbial composition especially in prolonged cultivation periods in comparison to European aspen plantations.

MATERIALS AND METHODS

Description of aspen and hybrid aspen stands and soil sampling

Soil in four hybrid aspen and four European aspen stands growing on former agricultural soils was analysed (Fig. 1). Information about the stands, tree age, and sampling time is given in Table 1. In each stand 10 soil samples (500 g) were

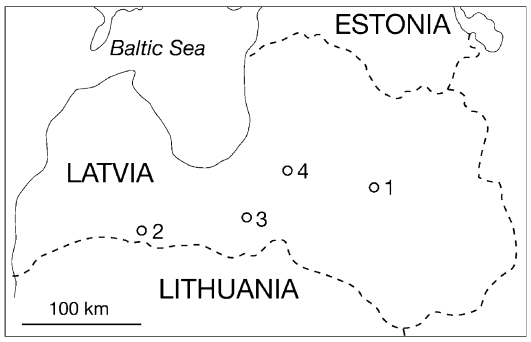

Fig. 1. Location of the studied stands. 1 – Forest Research Station 'Kalsnava', Kalsnava municipality; 2 – Forest Research Station 'Auce', Ukri municipality; 3 – private land, Iecava municipality; 4 – private land, Ropazi municipality.

Table 1. Characterization of the sampled aspen stands

Location	Year of planting and productivity		Characterization	Time of soil sampling
	Hybrid aspen	European aspen		
Forest Research Station 'Kalsnava', Kalsnava municipality	1964[a]	1966[a]	Hybrid aspen (1.1 ha) and European aspen (1.1 ha) in separate stands. Every five years offshoots are cut out	23.08.2010
Forest Research Station 'Auce', Ukri municipality	2000; 87 m^3 ha^{-1}[b]	2000; 39 m^3 ha^{-1}[b]	In parcels (5 × 5 trees), 1100 trees ha^{-1}	15.09.2010
Privately owned land, Iecava municipality	1998; 215 m^3 ha^{-1}[b]	1998; 60 m^3 ha^{-1}[b]	In parcels (3 × 5 trees), 2500 trees ha^{-1}	30.08.2011
Privately owned land, Ropazi municipality	2001[c]	2001[c]	In parcels (5 × 5 trees), 1100 trees ha^{-1}. European aspen clones were triploid	11.10.2011

[a] These data are not comparable due to the management activities that include tree crown forming.
[b] Average productivity estimated at the age of 12 years.
[c] Not estimated yet.

taken in the upper soil layer (0–15 cm) and 10 in the deeper soil layer (16–30 cm). In Kalsnava soil samples were taken diagonally through both stands. In other stands samples were taken randomly from various aspen clones planted in parcels in the middle of every parcel. All together 160 soil samples were analysed. Samples were placed in sterile plastic bags (*Nasco* WHIRL-PAK) and stored at +4 °C for a few days until the plate count of culturable microorganisms was made. After that the samples were stored at –20 °C.

Physical and chemical analyses of soil

Soil moisture content was determined according to the ISO 11465 standard method for every soil sample. The pH of the soil samples was measured in distilled water according to the method ISO 10390. Soil physical and chemical analyses of four soil samples from every aspen stand in three replicates were made in the laboratory of Forest Regeneration and Establishment Group of the Latvian State Forest Research Institute 'Silava'. The following methods were used: LVS ISO 11464

(2006) Soil quality – Pretreatment of samples for physico-chemical analysis, LVS ISO 11465 (2006) Soil quality – Determination of dry matter and water content on a mass basis – Gravimetric method, LVS ISO 11277 (2010) Soil quality – Determination of particle size distribution in mineral soil material – Method by sieving and sedimentation, LVS ISO 11261 (2002) Soil quality – Determination of total nitrogen – Modified Kjeldahl method, LVS ISO 10694 (2006) Soil quality – Determination of organic and total carbon after dry combustion (elementary analysis), LVS ISO 10693:1995 Soil quality – Determination of carbonate content – Volumetric method, LVS ISO 11466:1995 Soil quality – Extraction of trace elements soluble in aqua regia, LVS 398 (2002) Soil quality – Determination of total phosphorus. The content of humic acids was determined according to the method of Zaccone et al. (2009).

Analysis of culturable soil microorganisms

In order to estimate the number of colony forming units (CFU) of culturable filamentous fungi (CFF), yeasts, and bacteria by the plate count method, soil sample dilutions were prepared by adding 10 g of soil to 90 mL of sterile distilled water. Suspensions were homogenized on a horizontal shaker for 1 h. After that serial dilutions were prepared, and 0.1 mL of dilutions 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were analysed. Agarised tryptic soy (Biolife, Italy) medium (TSA) was used for the enumeration of saprophytic bacterial CFU. The incubation time was 3 days, temperature 20±2 °C. Agarised malt extract (MEA), 30 g/L, pH 5.5 (Biolife, Italy), was used as a growing medium that is favourable for fungi, yeasts, and maltose using bacteria. Rose Bengal agar (RBA) with chloramphenicol (Biolife, Italy) was used for the enumeration of fungi and yeasts. The incubation time for fungi was 5 days, temperature 20±2 °C. The number of CFU was expressed per gram of dry soil.

Genera of CFF were determined after 10 days of incubation according to morphological characteristics and light microscopy results.

Extraction of total soil DNA and quality control

Total soil DNA was extracted using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., USA), which uses harsh lysis for the DNA extraction. Samples (250 mg) were homogenized using a horizontal Mixer Mill Type MM 301 (Retsch, Germany) at a maximal speed of 30 Hz (1800 oscillations/min) for 10 min. The amount and purity of the DNA were determined spectrophotometrically using Ultrospec 3100 Pro (Amersham Biosciences, UK) at wavelengths of 230, 260, and 280 nm in order to assess the contamination with proteins and humic compounds (Yeates et al., 1998).

ARDRA of soil DNA

For the amplified ribosomal DNA gene analysis (ARDRA) the fungal rRNA gene region that contains two internal transcribed spacers (ITS) and the 5.8S rRNA gene (ITS1-5.8S-ITS2) was amplified with the primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990). In order to obtain the fingerprints of bacterial populations, the 16S region of bacterial ribosomal RNA was amplified with primers FORB and REVB (Edwards et al., 1989; Yeates et al., 1998).

The PCR reactions in Eppendorf Mastercycler Personal were carried out in 50 μL volume. The mixture contained 0.4 μL of Hot Start *Taq* DNA Polymerase, 5 μL of 10X Hot Start PCR Buffer, 5 μL of dNTP Mix, 2 mM each, 4 μL of 25 mM $MgCl_2$, 0.75 μL of Bovine Serum Albumin 20 mg/mL (all reagents from Fermentas, Lithuania), 1 μL of each 25 μM primer (OPERON Biotechnologies, Germany), 30.85 μL of sterile distilled water, and 1 μL of DNA template. The polymerase chain reaction (PCR) conditions were as follows: the initial denaturation step of 4 min at 95 °C, 40 s of denaturation at 95 °C, 40 s of annealing at 52 °C, 1 min of primer extension at 72 °C (30 cycles), and final extension 10 min at 72 °C.

For ARDRA analysis with restriction endonuclease *Bsu*RI (Chabrerie et al., 2003) the amplification products after the PCR were precipitated by 450 μL of 90% ethanol and 0.3 M sodium acetate (pH 5.0). The precipitated DNA was washed with 70% ethanol, air dried, dissolved in ddH_2O, and digested with *Bsu*RI (Fermentas, Lithuania). Restriction fragments were visualized in native 6% polyacrylamide gel electrophoresis using a Mighty Small^{TM} II (Hoefer, USA) unit. Gels were stained with ethidium bromide, photographed, and analysed with the software KODAK1D. For the calculation of the Shannon–Weaver diversity index (H') the following equation was used: $H' = -\sum p_j \log_2 p_j$, where p_j denotes relative intensity of an individual band (Gabor et al., 2003).

Sequencing of ribosomal DNA

A total of 221 isolates (88 sporulating and 133 white or black sterile mycelia, i.e. not sporulating when kept at +4 °C for several months) representing dominant filamentous fungi were isolated from the plates used for the enumeration of CFF and subcultured on MEA. Genomic DNA from approximately 0.25 g of mycelia was extracted using the method developed by Cenis (1992). The extracted DNA was amplified by PCR with primers ITS4 and ITS1F. The amplified DNA fragments from fungal isolates (101) from aspen stands in Kalsnava and Ukri were sequenced in CBS KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands, in the frame of the project EMbaRC. After PCR 5 μL of each amplified product was subjected to a sequencing protocol with BigDye Terminator Cycle Sequencing Kit (version 3.1, Applied Biosystems, USA) with both primers at CBS and only with primer ITS1F in Latvia. The sequencing of the samples from Iecava and Ropazi was performed at the Latvian Biomedical Research and Study Center. Double stranded sequences of PCR amplicons were assembled using Staden Package 1.6.0. Homology search was done against the National Centre for

Biotechnology Information GenBank nucleotide database using the Basic Local Alignment Search Tool or against the Q-bank database (www.q-bank.eu).

Taxonomic data based on sequenced ribosomal DNA ITS regions of the isolates were used to calculate Sørensen's community similarity index C_s using the following equation: $C_s = 2C/A + B$, in which A and B are the number of species in samples A and B, respectively, and C is the number of species shared by the two samples (Izzo et al., 2006).

Statistical analyses

The *F*-test, *t*-test ($\alpha = 0.05$), and correlation analysis were made with *Excel* (Microsoft, USA). The microbiological data (number of CFU of fungi and bacteria, total soil DNA amount, as well as the results of ARDRA) were analysed for every geographical location separately; for example, the data from the hybrid aspen stand in Kalsnava were compared with the data from the European aspen in Kalsnava. The ten soil samples were treated as one data set ($n = 10$).

Multiple regression analysis was made with the R package (R Development Core Team, 2009). In the regression models stands of European aspen were labelled with 0 and stands of hybrid aspens with 1. Since soil chemical analyses were made only for four soil samples from each geographical location, average values of microbiological data of 10 samples from the same soil depth were used in multiple regression analysis ($n = 4$). Due to co-linearity of total nitrogen and total carbon values C/N ratios were used in the regression models.

RESULTS AND DISCUSSION

Physical and chemical characterization of soil

Data on the element content, total nitrogen content, and soil pH at both analysed depths of all soil profiles are listed in Table 2. Information on soil texture and moisture content is given in Table 3. The European aspen and hybrid aspen stands in Iecava are growing on soil that is chemically and granulometrically different from the soils of all other geographically distinct sampling plots. The upper soil layers down to 30 cm contained organic material, the soil there had the highest calcium content (39.30–71.32 g kg^{-1} versus 0.19–7.48 g kg^{-1} in other sampling plots) and the highest total carbon content (350.8–380.4 g kg^{-1} versus 10.8–39.0 g kg^{-1} in other places). In several cases the level of a particular element was reduced in the soil samples from hybrid aspen in comparison to the soil samples from European aspen (Table 2). For example, total nitrogen content and potassium content were reduced in all sampling plots except in Iecava. An increased C/N ratio in the soil of the hybrid aspen stand was observed only in Kalsnava, probably due to the age of this stand. This is in line with other investigations showing that hybrid aspen litter has an increased C/N ratio in comparison to European aspen litter (Nikula et al., 2009) followed by carbon sequestration in the soil (Baum et al., 2009).

Table 2. Element content, C/N ratio, the content of humic acids ($n = 3$), and soil pH at both analysed depths in all experimental plots. Asterisks indicate statistically significant differences between the soil samples from hybrid aspen (HA) and European aspen (EA) stands or parcels at the respective depths

Plot & depth, cm	Ca, g kg^{-1}	Mg, g kg^{-1}	K, g kg^{-1}	C_{total}, g kg^{-1}	P, g kg^{-1}	N_{total}, g kg^{-1}	C/N ratio	Humic acids, g kg^{-1}	pH_{H2O}
Kalsnava, HA									
0–15	7.48±0.505*	1.53±0.103	0.42±0.019*	21.4±1.6*	0.22±0.04	1.0±0.10*	21	13.15±0.01*	6.62±0.44
16–30	0.19±0.013*	0.95±0.064*	0.50±0.023*	19.5±0.7	0.19±0.03	0.8±0.00*	23	36.29±0.02*	6.03±0.16
Kalsnava, EA									
0–15	0.47±0.032*	1.69±0.114	0.82±0.037*	31.3±0.6*	0.17±0.03	1.7±0.00*	19	20.33±2.69*	6.70±0.21
16–30	0.63±0.043*	1.64±0.111*	0.68±0.031*	20.6±0.2	0.16±0.03	1.2±0.00*	17	24.57±2.06*	6.31±0.64
Ukri, HA									
0–15	0.59±0.040*	2.10±0.142*	2.49±0.114*	20.3±1.2*	0.12±0.02*	1.3±0.10*	15	50.66±0.75*	7.12±0.07
16–30	0.56±0.038*	2.31±0.156	4.02±0.183*	10.8±0.4*	0.12±0.02*	0.8±0.00*	13	75.72±9.64*	6.97±0.28
Ukri, EA									
0–15	1.49±0.101*	2.84±0.192*	1.78±0.081*	39.00±3.3*	0.18±0.03*	2.7±0.10*	14	43.14±2.01*	7.01±0.37
16–30	1.10±0.074*	2.21±0.149	2.10±0.096*	29.00±0.3*	0.17±0.02*	2.1±0.10*	14	47.81±1.84*	7.12±0.19
Iecava, HA									
0–15	70.43±4.757*	3.79±0.256*	0.52±0.024	380.2±3.90*	0.36±0.06	22.1±1.50	17	71.99±8.20	6.51±0.13
16–30	71.32±4.817	3.98±0.269	0.56±0.026	364.4±3.70*	0.35±0.06	23.3±0.40	16	73.88±23.26	6.69±0.09
Iecava, EA									
0–15	39.30±2.654*	3.26±0.220*	0.51±0.023	350.8±1.7*	0.35±0.06	22.1±1.60	16	73.86±12.75	6.65±0.24
16–30	68.10±4.599	3.75±0.253	0.56±0.026	370.2±1.2*	0.33±0.06	22.4±2.70	17	77.30±7.65	6.69±0.25
Ropazi, HA									
0–15	0.30±0.020*	0.35±0.024*	0.41±0.019*	18.3±0.6*	0.10±0.02*	1.4±0.10*	13	15.88±0.24	6.23±0.08
16–30	0.41±0.028*	0.36±0.024*	0.40±0.018*	15.1±0.4*	0.11±0.02*	1.0±0.00*	15	15.51±1.31*	6.08±0.23
Ropazi, EA									
0–15	0.79±0.053*	0.84±0.057*	0.87±0.040*	35.6±3.2*	0.23±0.05*	2.2±0.20*	16	26.36±11.78	6.75±0.40
16–30	0.38±0.026*	0.51±0.034*	0.46±0.021*	27.3±1.2*	0.19±0.04*	1.7±0.10*	16	20.08±0.34*	6.29±0.19

Table 3. Granulometric content ($n = 3$), soil texture class and soil moisture content ($n = 10$) at both analysed depths in all experimental plots. Asterisks indicate statistically significant differences between the soil samples from hybrid aspen (HA) and European aspen (EA) stands or parcels at the respective depths

Plot & depth, cm	Clay, %	Silt, %	Sand, %	Soil texture class	Soil moisture content, %
Kalsnava, HA					
0–15	3.1 ± 0.1*	19.3 ± 0.9*	77.6 ± 2.1*	Loamy sand	25.74 ± 12.07
16–30	4.9 ± 0.2*	23.6 ± 1.1*	71.4 ± 1.9*	Sandy loam	26.95 ± 12.65
Kalsnava, EA					
0–15	8.9 ± 0.4*	25.0 ± 1.1*	66.1 ± 1.8*	Sandy loam	21.72 ± 2.07
16–30	8.1 ± 0.3*	28.2 ± 1.3*	63.8 ± 1.7*	Sandy loam	17.30 ± 1.94
Ukri, HA					
0–15	17.2 ± 0.7	28.3 ± 1.3	54.4 ± 1.5	Sandy loam	15.61 ± 7.58
16–30	24.7 ± 1.0*	28.5 ± 1.3	46.7 ± 1.3*	Loam	12.27 ± 2.45
Ukri, EA					
0–15	17.5 ± 0.7	28.7 ± 1.3	53.8 ± 1.4	Sandy loam	24.96 ± 19.48
16–30	15.8 ± 0.6*	31.7 ± 1.4	52.5 ± 1.4*	Sandy loam	14.57 ± 2.97
Iecava, HA					
0–15	–[a]	–	–	–	50.06 ± 3.85
16–30	–	–	–	–	49.28 ± 7.02
Iecava, EA					
0–15	–	–	–	–	51.85 ± 3.86
16–30	–	–	–	–	52.75 ± 3.85
Ropazi, HA					
0–15	2.8 ± 0.1*	18.9 ± 0.9*	78.3 ± 2.1*	Loamy sand	22.49 ± 2.50
16–30	1.9 ± 0.1*	18.3 ± 0.8*	79.8 ± 2.2*	Loamy sand	16.32 ± 1.98
Ropazi, EA					
0–15	4.5 ± 0.2*	24.4 ± 1.1*	71.0 ± 1.9*	Sandy loam	25.37 ± 8.39
16–30	4.5 ± 0.2*	23.0 ± 1.0*	72.5 ± 2.0*	Sandy loam	16.66 ± 4.85

[a] The soil of these sampling plots was drained peat soil that contained only organic material at a depth of 0–30 cm.

Analysis of culturable microorganisms

The count of culturable microorganisms on three microbial media is given in Fig. 2a–e. The following statistically significant differences between the soils of hybrid aspen and European aspen stands were detected: a significantly higher number of bacterial CFU on TSA in the European aspen stand at Kalsnava ($F = 1.13$, $p = 0.02$; 2.14×10^6 CFU g^{-1} of dry soil versus 1.56×10^6 CFU g^{-1} of dry soil), significantly higher numbers of yeasts and maltose utilizing bacteria on MEA in the European aspen stand at Kalsnava ($F = 1.52$, $p = 0.003$; 4.86×10^6 CFU g^{-1} of dry soil versus 1.80×10^6 CFU g^{-1} of dry soil), and significantly higher numbers of filamentous fungi CFU on MEA at a depth of 16–30 cm in the soil of the hybrid aspen clones at Ropazi ($F = 12.27$, $p = 0.007$; 6.41×10^4 CFU g^{-1} of dry soil versus 3.23×10^4 CFU g^{-1} of dry soil).

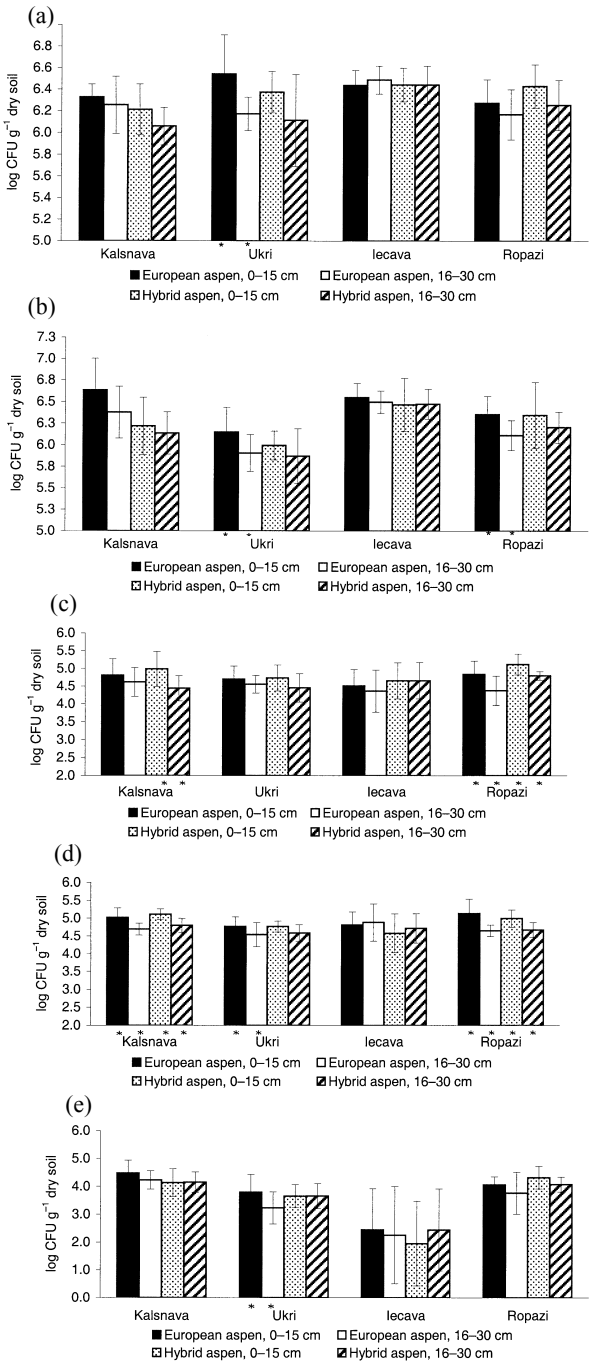

Fig. 2. Number of CFU of bacteria on TSA (a), yeasts and maltose utilizing bacteria on MEA (b), fungi on MEA (c), fungi on RBA (d), and yeasts on RBA (e) at both sampling depths in all aspen stands (±SD; $n = 10$). Results were obtained after three (in the case of bacteria) or five days (in the case of fungi) of incubation at 20±2 °C. Asterisks indicate statistically significant differences ($p < 0.05$) between the sampling depths.

The total number of soil bacteria fluctuated from 5.37 to 6.99 log CFU g^{-1} of dry soil, which is similar to the total number of bacteria in agricultural soils, where it ranges from 5.10 to 7.85 log CFU g^{-1} of dry soil (Grantina et al., 2011a). The number of yeasts and maltose utilizing bacteria on MEA ranged from 5.34 to 6.71 log CFU g^{-1} of dry soil and the total number of CFF was from 3.93 to 5.92 log CFU g^{-1} of dry soil. These numbers are on average slightly higher than in agricultural soil where they fluctuate from 3.72 to 6.14 log CFU g^{-1} of dry soil and from 2.53 to 4.89 log CFU g^{-1} of dry soil, respectively (Grantina et al., 2011a), being more similar to forest soils where the level of CFF is 3.24–5.59 log CFU g^{-1} of dry soil in spruce forest (Grantina et al., 2012) and 3.58–5.08 log CFU g^{-1} of dry soil in mixed and pine forests (Grantina et al., 2011b).

In general the estimated CFU values represent the minimal number for CFF, because the method does not differentiate between colonies formed by single fungal spores and colonies formed by multicellular pieces of mycelium (Matthies et al., 1997). Moreover, as only a small number of bacteria (Torsvik et al., 1996) and fungi (Bridge & Spooner, 2001) are culturable, we used molecular biology methods to target also unculturable microbial populations.

Taxonomic analysis of soil fungal populations

According to the plate count method, the dominant genera of filamentous fungi were *Penicillium*, *Trichoderma*, *Paecilomyces*, *Mortierella*, and *Mucor* (Table 4). No statistically significant differences were observed between the soils of hybrid

Table 4. Average percentage of the representatives of five dominant fungal genera in the experimental plots (number of soil samples from each depth $n = 10$)

Experimental plot	Depth, cm	*Penicillium* spp., %		*Mortierella* spp., %		*Trichoderma* spp., %		*Paecilomyces* spp., %		*Mucor* spp., %	
		RBA	MEA	RBA	MEA	RBA	MEA	RBA	MEA	RBA	MEA
Kalsnava,	0–15	40.83	25.46	0.00	0.68	8.22	6.74	4.68	40.73	1.14	1.15
European aspen	16–30	32.47	40.34	5.41	8.42	1.71	13.49	11.85	0.00	0.33	19.88
Kalsnava, hybrid	0–15	37.78	19.15	0.87	9.31	0.65	0.71	0.00	38.14	1.18	0.68
aspen	16–30	24.25	13.56	0.15	3.39	0.16	0.00	0.00	0.00	2.25	3.39
Ukri, European	0–15	19.97	36.39	4.24	8.16	1.21	7.53	12.78	0.00	0.43	0.25
aspen	16–30	20.01	45.34	13.63	0.05	1.70	0.05	4.95	0.00	1.73	0.82
Ukri, hybrid	0–15	30.62	29.88	14.89	1.36	1.14	1.24	0.00	0.00	1.08	0.13
aspen	16–30	12.04	32.86	0.88	9.28	1.13	2.31	35.65	0.00	3.24	0.02
Iecava, European	0–15	24.76	14.37	5.87	18.30	0.87	0.25	29.37	15.70	1.01	1.04
aspen	16–30	16.05	32.59	10.95	10.01	0.17	2.36	3.04	8.84	1.03	1.58
Iecava, hybrid	0–15	22.42	14.52	4.32	0.62	1.21	11.53	16.93	13.38	0.97	2.30
aspen	16–30	25.44	20.73	2.57	6.79	2.40	1.87	1.03	2.66	1.11	1.53
Ropazi, European	0–15	29.45	37.14	5.86	0.98	2.03	5.92	15.42	11.24	0.78	1.23
aspen	16–30	32.73	13.04	1.86	10.48	4.05	1.03	17.60	0.85	9.36	2.16
Ropazi, hybrid	0–15	19.69	45.80	4.99	7.19	12.47	2.38	8.69	6.53	0.85	2.45
aspen	16–30	10.38	17.22	10.30	5.06	9.90	3.24	1.66	31.40	0.42	1.54

aspen and European aspen stands. Probably the reason was the fluctuation of results among 10 samples from the same plot and the same soil depth (Table 4). The members of the *Penicillium* genus ranged from 10.38% to 40.83% on RBA and from 13.04 to 45.80% on MEA (Table 4). *Mortierella* spp. constituted from zero to 14.89% on RBA and from 0.05% to 18.30% on MEA. Representatives of the *Trichoderma* genus ranged from 0.16% to 12.47% on RBA and from zero to 13.49% on MEA. The variation in the abundance of *Paecilomyces* spp. was from zero to 35.65% on RBA and from zero to 40.73% on MEA. The abundance of *Mucor* spp. varied from 0.33% to 9.36% on RBA and from 0.02% to 19.88% on MEA. Representatives of these five dominant genera constituted from 26.81% to 65.60% of all culturable fungi on RBA and from 20.34% to 82.13% on MEA. Less abundant genera were *Talaromyces*, *Umbelopsis*, *Metarhizium*, *Beauveria*, and *Acremonium*. Sterile white and black mycelia were observed as well.

The list of fungal genera determined according to morphological characters or sequencing results is given in Table 5. From 221 isolates sequenced 93.66% were Ascomycota (representing 58 genera), 4.98% Zygomycota (three genera), and only 1.36% Basidiomycota (one genus); all these together included 95 species. The largest part of the fungi were saprophytic: *Acremonium felinum*, *Acremonium strictum* (Watanabe, 2002), *Cladosporium cladosporioides*, *Mucor hiemalis*, *Paecilomyces carneus*, *Penicillium montanense* (Jayasinghe & Parkinson, 2008), *Penicillium* spp. (Baldrian et al., 2011), etc. Several identified species are entomopathogenic according to the literature: *Beauveria geodes*, *Tolypocladium geodes* (Santos et al., 2011), *Metarhizium anisopliae* (Leger et al., 1992), *Paecilomyces carneus*, *P. marquandii* (Sevim et al., 2010), *Isaria fumosorosea* (Ayala-Zermeño et al., 2011), *Lecanicillium kalimantanense* (Sukarno et al., 2009). *Bionectria ochroleuca* is associated with soil mites (Renker et al., 2005). Certain species are characterized as plant pathogens: *Gibberella pulicaris* (Desjardins & Gardner, 1991), *Leptosphaeria* sp. (Pedras, 2011), *Neonectria radicicola* (Halleen et al., 2004), or pathogens of soil nematodes: *Plectosphaerella cucumerina* (Atkins et al., 2003). Several species are associated with aspen leaves and roots: *Sagenomella diversispora* (Samson et al., 2011), *Lophiostoma* sp. (Albrectsen et al., 2010), or soil of aspen stands: *Trichoderma rossicum* (Friedl & Druzhinina, 2012). Fourteen species were detected in the investigation of microfungal communities of white spruce (*Picea glauca* (Moench) Voss) and trembling aspen (*P. tremuloides* Michx.) logs at different stages of decay in disturbed and undisturbed sites in boreal mixedwood forests of Alberta, Canada (Lumley et al., 2001) (last column in Table 5). Several species have been recorded to be associated with other tree species than aspens. For example, *Neonectria macrodidyma* was isolated as a root pathogen from the roots of nursery cultivated *Pinus sylvestris* L. (Menkis & Burokiene, 2012). Six species have been isolated in the rhizosphere of *Salix* spp.; these include *Humicola fuscoatra*, *Mortierella alpina*, *Volutella ciliata*, *Penicillium janthinellum*, and *Plectosphaerella cucumerina*. The abundance of the last two species correlates with the acid-phosphatase and arylsulphatase activity in the rhizosphere and is so related with the phosphorus and sulphur cycle in the soil

Table 5. List of fungal genera determined in the soil of hybrid aspen (HA) and European aspen (EA) stands according to morphological characters (indicated by x) or ribosomal DNA sequencing (indicated by total number of isolates and GenBank accession numbers of the homologue sequence)

Species	Kalsnava		Ukri		Iecava		Ropazi		Homologue sequence, NCBI acc. No.	No. in MSCL[a]	Detected in Lumley et al., 2001
	HA	EA	HA	EA	HA	EA	HA	EA			
Ascomycota											
Acremonium felinum (Marchal) Kiyuna, An, Kigawa & Sugiy.		1	3	3					AB540562.1	1058	–
Acremonium strictum W. Gams		1	2	1					U57671.1	1063, 1064, 1120	+
Acremonium sp.		x							–	–	–
Alternaria sp.				1	1		1		GU934500.1		–
Aporospora terricola J.C. Krug & Jeng			1						AF049088.1	–	–
Arthrinium sacchari (Speg.) M.B. Ellis								1	EU579803.1	–	+
Aureobasidium sp.		1							GQ906942.1	1116	–
Auxarthron umbrinum (Boud.) G.F. Orr & Plunkett				1					FR718876.1	–	–
Beauveria geodes (W. Gams) Arx				x	x	x	1		U19037.1	1187	–
Beauveria sp.				x	x	x	x	x	–	–	–
Bionectria levigata Schroers					1				AF210680.1	1202	–
Bionectria ochroleuca (Schwein.) Schroers & Samuels					2		1	1	GU934503.1	1190, 1196, 1247	–
Botryotinia fuckeliana (de Bary) Whetzel	1								HM989942.1	–	–
Cadophora finlandica (C.J.K. Wang & H.E. Wilcox) T.C. Harr. & McNew							2		EF093179.1	1248, 1259	–
Cladosporium cladosporioides (Fresen.) G.A. de Vries								1	HQ832794.1	–	+
Clonostachys divergens Schroers								1	GU934587.1	1268	–

Continued overleaf

Table 5. *Continued*

Species	Kalsnava		Ukri		Iecava		Ropazi		Homologue sequence, NCBI acc. No.	No. in MSCL[a]	Detected in Lumley et al., 2001
	HA	EA	HA	EA	HA	EA	HA	EA			
Dokmaia sp.			2	1					HQ631068.1	1134	–
Emericella bicolor M. Chr. & States/*Aspergillus bicolor* M. Chr. & States					1				EF652511.1	1194	–
Emericella foeniculicola Udagawa						1			AB249011.1	1192	–
Eremomyces langeronii (Arx) Malloch & Sigler					1				AB128973.1	1199	–
Eucasphaeria capensis Crous				1			1	2	GU934520.1	1182, 1184, 1232	–
Exophiala salmonis J.W. Carmich.		1	1	1			1		GU586858.1	1114, 1117, 1124, 1260	+
Fusarium merismoides var. merismoides Corda					1				EU860057.1	1204	–
Fusarium oxysporum Schltdl.			1						GU445377.1	1105	–
Fusarium sp.		x				1			HQ731631.1	1199	–
Geomyces pannorum (Link) Sigler & J.W. Carmich.	1								DQ189229.1	1130	+
Geomyces destructans Blehert & Gargas			1	1					GU999986.1	1113	–
Geomyces sp.				1					DQ402527.1	1088	–
Gibberella pulicaris (Fr.) Sacc.				1					FJ481029.1	1143	–
Gibellulopsis nigrescens (Pethybr.) Zare, W. Gams & Summerb.					3	1	1	5	HQ115693.1	1189, 1191, 1205, 1250, 1266, 1267, 1269	–

Table 5. *Continued*

Species	Kalsnava		Ukri		Iecava		Ropazi		Homologue sequence, NCBI acc. No.	No. in MSCL[a]	Detected in Lumley et al., 2001
	HA	EA	HA	EA	HA	EA	HA	EA			
Gliomastix murorum (Corda) S. Hughes					1				AB540556.1	1235	–
Humicola fuscoatra Traaen						1			AB625589.1	–	+
Humicola sp.		x							–	–	–
Hyalodendriella betulae Crous								1	EU040232.1	1243	–
Hypocrea koningii Lieckf., Samuels & W. Gams/*Trichoderma koningii* Oudem.		2							AJ301990.1	1131	+
Hypocrea lixii Pat.							2		AY605716.1, HQ259311.1	1230, 1245	–
Hypocrea pachybasioides Yoshim. Doi	1								GU934589.1	1127	–
Hypocrea viridescens Jaklitsch & Samuels/*Trichoderma viridescens* (A.S. Horne & H.S. Will.) Jaklitsch & Samuels		2							GU566274.1	–	–
Ilyonectria europaea A. Cabral, Rego & Crous							1	1	JF735294.1	1241	–
Ilyonectria rufa A. Cabral & Crous								1	JF735278.1	–	–
Isaria fumosorosea Wize								1	JF792885.1	1239	–
Kernia pachypleura Malloch & Cain								1	DQ318208.1	1186	–
Lecanicillium kalimantanense Kurihara & Sukarno								1	AB360356.1	1270	–
Lecanicillium sp.								1	AB378528.1	1242	–
Leptosphaeria conferta Niessl ex Sacc.								1	AF439459.1	1264	–
Leptosphaeria maculans (Desm.) Ces. & De Not.						1			M96663.1	1196	–
Leptosphaeria sp.		2	2			2			AY336132.1	1062, 1128	–

Continued overleaf

Table 5. *Continued*

Species	Kalsnava		Ukri		Iecava		Ropazi		Homologue sequence, NCBI acc. No.	No. in MSCL[a]	Detected in Lumley et al., 2001
	HA	EA	HA	EA	HA	EA	HA	EA			
Leptospora rubella				1					AF383951.1	–	–
Leucostoma persoonii (Nitschke) Höhn.					1				HM061319.1	1193	–
Lophiostoma sp.					1				HM116744.1	–	–
Metarhizium anisopliae (Metschn.) Sorokīn	x	1 x		1 x		1 x		x	HM055436.1	1112	–
Nectria lugdunensis J. Webster		1							DQ247778.1	1101	–
Nectria mariannaeae Samuels & Seifert			1						EU273515.1	1048	–
Nectria vilior Starbäck					1				U57673.1	1198	–
Neonectria macrodidyma Halleen, Schroers & Crous							1	1	HM036602.1	–	–
Neonectria radicicola (Gerlach & L. Nilsson) Mantiri & Samuels			1						AJ875336.1	1123	–
Neonectria ramulariae Wollenw.							2		JF735314.1	1262, 1263	–
Paecilomyces carneus (Duché & R. Heim) A.H.S. Br. & G. Sm.	1	2				1			AB258369.1	1140, 1126, 1188	+
Paecilomyces marquandii (Massee) S. Hughes			3	2	1	2	1	2	AB099511.1, AB114223.1, FR799470.1	1061, 1121, 1141, 1151, 1236, 1237, 1255, 1257	–
Paecilomyces sp.	x	x	x	x	x	x	x	x	–	–	–
Paraconiothyrium sporulosum (W. Gams & Domsch) Verkley			1						GU566257.1	1059	–

Table 5. *Continued*

Species	Kalsnava		Ukri		Iecava		Ropazi		Homologue sequence, NCBI acc. No.	No. in MSCL[a]	Detected in Lumley et al., 2001
	HA	EA	HA	EA	HA	EA	HA	EA			
Penicillium brasilianum Bat.								1	HM469396.1	1229	–
Penicillium canescens Sopp	2	1	7	1	1		7	7	FJ439586.1, FJ791141.1, AY373901.1, JF311911.1	1091, 1093, 1095, 1104, 1107, 1109, 1110, 1119, 1208–1216, 1223–1227	–
Penicillium corylophilum Dierckx	1								GU566277.1	1135	–
Penicillium janthinellum Biourge		1							AB293968.1	1097	–
Penicillium montanense M. Chr. & Backus								1	HQ157959.1	1228	–
Penicillium roseopurpureum Dierckx					1	2			JF311930.1, JN246027.1	1218–1220	–
Penicillium swieciickii K.M. Zalessky						1		3	AJ608946.1	1217, 1222, 1253	–
Penicillium sp.	1						2		GU446648.1	–	–
Penicillium sp.	x	x	x	x	x	x	x	x	–	–	–
Plectosphaerella cucumerina (Lindf.) W. Gams			2						AJ492873.1	1103	–
Pochonia suchlasporia (W. Gams & Dackman) Zare & W. Gams					1			2	AB214658.1	1192, 1240	–

Continued overleaf

Table 5. *Continued*

Species	Kalsnava		Ukri		Iecava		Ropazi		Homologue sequence, NCBI acc. No.	No. in MSCL[a]	Detected in Lumley et al., 2001
	HA	EA	HA	EA	HA	EA	HA	EA			
Podospora appendiculata (Auersw. ex Niessl) Niessl								1	AY999126.1	1201	–
Pseudallescheria fimeti (Arx, Mukerji & N. Singh) McGinnis, A.A. Padhye & Ajello							1		AY879799.1	–	–
Pseudeurotium bakeri C. Booth				1					GU934582.1	1133	–
Pyrenochaeta acicola (Moug. & Lév.) Sacc.								1	*	1251	–
Pyrenochaeta inflorescentiae Crous, Marinc. & M.J. Wingf.			1				1		GU586851.1	1149	–
Rhizopycnis vagum D.F. Farr	1								HQ610506.1	–	–
Sagenomella diversispora (J.F.H. Beyma) W. Gams		1							GQ169318.1	1111	–
Scedosporium apiospermum Sacc. ex Castell. & Chalm.			1						AB567756.1	1051	–
Scytalidium lignicola Pesante								1	FJ914697.1	–	+
Talaromyces ucrainicus Udagawa						x	x	1 x	AY533695.1	1231	–
Tetracladium setigerum (Grove) Ingold							1		FJ000374.1	1265	–
Trichocladium asperum Harz	1		1						HQ115689.1, AY706336.1	1108	–
Trichocladium opacum (Corda) S. Hughes				1					AM292049.1	958	–
Trichoderma hamatum (Bonord.) Bainier							3		JN542526.1	1233, 1234	+
Trichoderma rossicum Bissett, C.P. Kubicek & Szakács							1	1	DQ083024.1, EU280089.1	1183, 1246	–
Trichoderma tomentosum Bissett				1					AY605737.1	–	–
Trichoderma sp.		x	x	x	x	x	x	x	–	–	–
Verticillium dahliae Kleb.					1			1	HQ839784.1	1197	–

Table 5. *Continued*

Species	Kalsnava		Ukri		Iecava		Ropazi		Homologue sequence, NCBI acc. No.	No. in MSCL[a]	Detected in Lumley et al., 2001
	HA	EA	HA	EA	HA	EA	HA	EA			
Verticillium sp.		x							–	–	–
Volutella ciliata (Alb. & Schwein.) Fr.			1			1			GU586855.1, HQ897802.1	1203	+
Wardomyces inflatus (Marchal) Hennebert								1	FJ946485.1	1252	+
Zalerion varium Anastasiou	1								AJ608987.1	1118	–
Zygomycota											
Mortierella alpina Peyronel	1	1						2	AJ271629.1, FJ478130.1, JN943023.1	1098, 1254, 1271	+
Mortierella clonocystis W. Gams						1			HQ630318.1	1200	–
Mortierella globulifera O. Rostr.							1		JN943800.1	1244	–
Mortierella sp.		3	1						FJ810149.1, FJ810151.1, DQ093725.1	1052	–
Mortierella sp.	x	x	x	x	x	x	x	x	–	–	–
Mucor hiemalis Wehmer							1		EU484263.1	1181	+
Mucor sp.	x	x	x	x	x	x	x	x	–	–	–
Umbelopsis sp.	x		x		x			x	–	–	–
Basidiomycota											
Trichosporon porosum (Stautz) Middelhoven, Scorzetti & Fell								1	AJ608971.1	–	–
Trichosporon sp.		2							FJ439589.1	–	–

[a] Microbial Strain Collection of Latvia.
* According to the Q-bank database.

(Baum & Hrynkiewicz, 2006). Several species have been isolated from the soil of boreal mixedwood forests in Canada (*P. janthinellum*, *Trichoderma hamatum*, *T. koningii*, *Geomyces pannorum*, *M. hiemalis*, *P. carneus* (De Bellis et al., 2007)) and/or in the soil of birch stands in central Finland (*M. hiemalis*, *Paecilomyces carneus*, and *Penicillium montanense* (McLean & Huhta, 2002)).

The most common fungal species in the list of sequenced isolates was *Penicillium canescens* with a total of 26 isolates (11.76% of all sequenced isolates). This particular species has not been detected in the other investigations of forest soil microflora mentioned in the previous paragraph. Together with other *Penicillium* species (40 isolates in total) this genus constituted 18.10% of the sequenced isolates. These results are in line with the results of the plate count, where the most abundant genus was *Penicillium*. Other common genera were *Paecilomyces* (15 isolates) and *Hypocrea*/*Trichoderma* (13 isolates) in all sampling plots, *Acremonium* (11 isolates, all from the sampling plots in Kalsnava and Ukri), *Mortierella* (10 isolates, in all sampling plots), *Gibellulopsis* (10 isolates, isolated as black sterile mycelia only in the sampling plots in Iecava and Ropazi), and *Leptosphaeria* (8 isolates, isolated as grey sterile mycelia in Kalsnava, Ukri, and Iecava).

Sørensen's community similarity indices C_s between each plot of hybrid aspen and European aspen are given in Table 6. The highest Sørensen's community similarity index between soil samples of the hybrid aspen stand and the European aspen stand was at Ukri – 0.39, in other sampling plots it ranged from 0.21 to 0.26. Indices C_s among all sampling plots were lower: from 0.07 (nearly complete dissimilarity between Ropazi and Kalsnava) to 0.22 (between Ukri and Kalsnava) (Table 7). The value of C_s between the fungal populations of all hybrid aspen

Table 6. Total number of fungal species, number of shared species, and values of Sørensen's community similarity indices C_s between each plot of hybrid aspen (HA) and European aspen (EA)

Sampling plot (total number of species)	Kalsnava HA (12)	Ukri HA (19)	Iecava HA (14)	Ropazi HA (23)
Kalsnava EA (15)	C_s = 0.22 (3 shared species)			
Ukri EA (17)		C_s = 0.39 (7 shared species)		
Iecava EA (15)			C_s = 0.21 (3 shared species)	
Ropazi EA (31)				C_s = 0.26 (7 shared species)

Table 7. Total number of fungal species, number of shared species, and values of Sørensen's community similarity indices C_s among all sampling plots

Sampling plot (total number of species)	Ukri (36)	Iecava (29)	Ropazi (54)
Kalsnava (27)	C_s = 0.22 (7 shared species)	C_s = 0.14 (4 shared species)	C_s = 0.07 (3 shared species)
Ukri (36)		C_s = 0.18 (6 shared species)	C_s = 0.11 (5 shared species)
Iecava (29)			C_s = 0.19 (8 shared species)

stands and all European aspen stands is 0.29 (21 shared species from 68 species of hybrid aspen stands and 78 species from European aspen stands).

All calculated Sørensen's indices revealed that the fungal populations in the analysed sampling plots were quite different from one another. This is in line with an investigation in Lithuania about fungal populations in the soil of seven polluted sites, where the highest value of Sørensen's indices was 0.37 although the total number of identified species was 158 (Pečiulytė & Dirginčiutė-Volodkienė, 2010).

Microbial diversity in soil based on ARDRA analysis

The amount of soil total DNA varied remarkably among sampling plots. The lowest amount of extracted DNA was in the sampling plots at Kalsnava and the highest amount was in the soil samples from Iecava (Fig. 3). Diversity indices calculated from the ARDRA data are given in Fig. 4. There were no statistically significant differences in the fungal and bacterial diversity between the two soil depths. For further statistical analyses the data from both soil depths were combined, but there were no significant differences in microbial diversity between the soil in the stands of hybrid aspen and the soil in the stands of European aspen. The only difference that approached the statistical significance was observed in the case of Ropazi: the average fungal diversity index H' was 2.33 in the hybrid aspen stand versus 2.01 in the European aspen stand (F = 1.27; p = 0.08). In this sampling plot statistically significant differences between the number of fungal CFU on MEA were observed at a depth of 10–30 cm comparing the soils from the hybrid aspen and European aspen stands. It was further corroborated by statistically different fungal diversity H' values of this soil level: – 2.55 versus 2.02 (F = 1.33; p = 0.03). In the case of fungal diversity versus bacterial diversity the difference among all four sampling plots was much greater than in the case of CFU numbers: significantly lower fungal diversity indices were obtained from the samples of Ukri in comparison with the samples of Iecava.

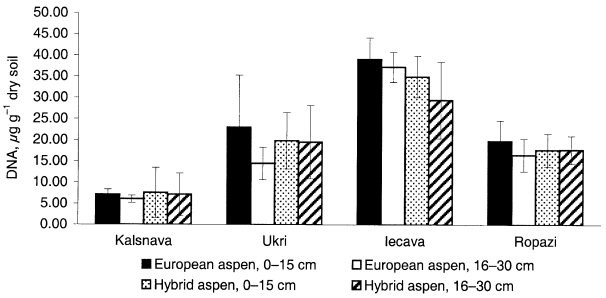

Fig. 3. Amount of total soil DNA in all sampling plots and at both analysed depths (± SD; $n = 10$).

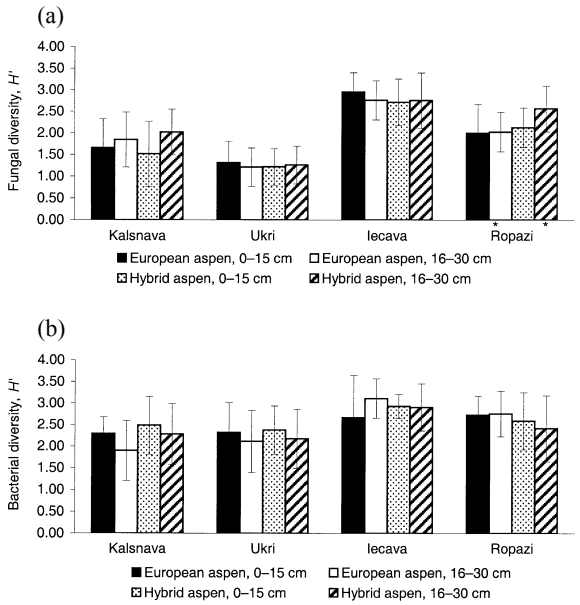

Fig. 4. Fungal (a) and bacterial (b) diversity indices H' in all sampling plots and at both analysed depths (± SD; $n = 10$). Asterisks indicate statistically significant differences ($p < 0.05$) between the sampling depths.

Multiple regression analyses of the impact of soil chemical characteristics on the microbial abundance and diversity

Considering that according to the *t*-test there were only a few statistically significant differences between the soil microbial populations of hybrid aspen and European aspen stands, soil chemical variables were included in multiple regression models in order to determine what parameters in general have a significant impact on the analysed soil microorganisms and on the amount of total soil DNA. The results of multiple regression analyses are summarized in Table 8. As it was

Table 8. Results of multiple regression analyses (*p*-values)

Factor	Total number of bacteria on TSA	Yeasts and maltose utilizing bacteria on MEA	Filamentous fungi on MEA	Yeasts on RBA	Filamentous fungi on RBA	Total amount of soil DNA	Fungal diversity, *H′*	Bacterial diversity, *H′*
Soil sampling depth	<0.0001[a]	NS	NS	NS	NS	NS	NS	NS
Aspen type	NS	NS	0.0005	NS	NS	NS	NS	NS
Soil moisture content	NS	<0.0001	0.0007	<0.0001	0.0005	<0.0001	<0.0001	NS
Soil pH	NS	NS	0.002	NS	0.02	NS	<0.0001[a]	0.02
Calcium content	NS	NS	NS	NS	NS	NS	NS	<0.0001
Magnesium content	0.0006	NS	NS	NS	0.003[a]	NS	NS	0.001[a]
Potassium content	0.0004[a]	NS	NS	NS	0.01	NS	NS	NS
Phosphorus content	0.02[a]	NS	NS	NS	0.046[a]	NS	NS	NS
C/N ratio	0.002[a]	NS	0.04[a]	NS	0.002	0.002[a]	0.02[a]	NS
Humic acids	NS	0.004[a]	<0.0001[a]	<0.004[a]	0.006[a]	0.03[a]	NS	NS
Multiple R^2	0.90	0.74	0.87	0.74	0.94	0.92	0.93	0.84
p-Value of the model	0.0001	0.0002	0.0003	0.0002	0.001	<0.0001	<0.0001	<0.0001

NS – not significant; [a] – negative impact.

detected with the *t*-test, the aspen type significantly affected the number of filamentous fungi on MEA but not the total number of bacteria on TSA. Although the soil pH values were similar in all sampling plots, the regression models showed that soil pH significantly influenced the number of filamentous fungi on MEA and RBA, and fungal and bacterial diversity indices H'. Of all the soil variables the most important were the C/N ratio and humic acid content. The C/N ratio values ranged from 13 to 23, and higher values of this ratio correlated with lower values of several microbiological variables such as the total number of bacteria on TSA, the number of filamentous fungi on MEA, the total amount of soil DNA, and fungal diversity. The concentration of humic acids in the soil negatively influenced the number of yeasts, maltose utilizing bacteria, and filamentous fungi on MEA, the number of yeasts and fungi on RBA, and the total amount of soil DNA. A similar regularity of the occurrence of a smaller number of yeasts in the soil layers with more humus was observed in the investigation of Priha et al. (2001) of soil microbial populations in pine, spruce, and birch stands in Finland.

CONCLUSIONS

1. In general, only a minor effect of hybrid aspen on soil microbial communities was observed. Our results show that during long cultivation periods (more than 40 years as in the case of the sampling plot at Kalsnava) hybrid aspens reduce the number of culturable bacteria, probably due to the increased C/N ratio in the soil. The number of culturable filamentous fungi was significantly increased only in one sampling plot (Ropazi) of hybrid aspen in the soil sampled at a depth of 16–30 cm and only on one microbiological cultivation medium. The same trend in the fungal diversity was detected in the same sampling plot also with molecular methods estimated by Shannon–Weaver diversity indices.
2. Hybrid aspen stands did not affect any other analysed characteristics of the soil microbial population, such as the number of yeasts and maltose utilizing bacteria on MEA, the number of yeasts and filamentous fungi on RBA, the total amount of soil DNA, the fungal and bacterial diversity estimated by the methods of molecular genetics, and species composition of filamentous fungi. So it is safe to cultivate hybrid aspen (*Populus tremuloide*s Michx. × *P. tremula* L.) as a short rotation forest tree species in the Baltic region if the abundance and diversity of saprophytic soil microorganisms is considered.

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Mulla mikroorganismide arvukuse ja mitmekesisuse võrdlus hariliku haava (*Populus tremula* L.) ning hübriidhaava (*Populus tremuloide*s Michx. × *P. tremula* L.) puistutes

Lelde Grantina-Ievina, Dace Saulite, Martins Zeps, Vizma Nikolajeva ja Nils Rostoks

Lühikese raieringiga majandatavaid puuliike kasutatakse maailma metsanduses üha enam. Balti regiooni kliimatingimustes on hübriidhaab (*Populus tremuloide*s Michx. × *P. tremula* L.) osutunud selleks otstarbeks sobilikuks. Lühikese raieringiga puuistandike kasvatamine endistel põllumuldadel erineb tavapõllumajandusest vähenenud maaharimise ja suurenenud toitainenõudluse poolest, mis pikas perspektiivis võib põhjustada muutusi mulla mikroobikooslustes. Käesoleva uurimuse eesmärgiks oli võrrelda mulla mikroobikooslusi hübriidhaava ja hariliku haava (*P. tremula* L.) puistutes neljal proovialal, kus haabade vanus jäi vahemikku 10 kuni 46 aastat. Mulla mikroobikoosluste arvukuse ja mitmekesisuse hindamiseks kasutati nende loendamist (plaadikülv kolmel erineval tardsöötmel) ning molekulaarseid meetodeid (PCR, ARDRA, seene isolaatide molekulaarne identifitseerimine). Tulemused näitasid, et hübriidhaava pikaaegse kasvatamise tagajärjel väheneb kultiveeritavate bakterite arvukus. Kultiveeritavate niitjate seente arvukus oli statistiliselt usaldusväärselt suurenenud vaid ühe hübriidhaava katseala mullas sügavusel 16–30 cm ja ainult ühel mikrobioloogilisel söötmel. Seda kinnitas mainitud katsealal ka molekulaarsete meetoditega määratud seente mitmekesisus, mida hinnati Shannon-Weaveri mitmekesisuse indeksiga. Teisi mulla mikroobikoosluse tunnuseid (pärmi ja maltoosi tarvitavate bakterite arvukus virdeagaril, pärmide ning niitjate seente arvukus Rose-Bengali tardsöötmel, kogu mullast eraldatud DNA, molekulaarbioloogiliste meetoditega leitud seente ja bakterite mitmekesisus ning niitjate seente liigiline koosseis) ei olnud hübriidhaabade kasv usaldusväärselt mõjutanud. Määratud niitjad seened esindasid järgmisi perekondi: *Acremonium*, *Exophiala*, *Geomyces*, *Gibellulopsis*, *Gibberella*, *Hypocrea*/*Trichoderma*, *Leptosphaeria*, *Metarhizium*, *Mortierella*, *Nectria*, *Paecilomyces*, *Penicillium*, *Trichosporon* ja teised. Peamise järeldusena leiti, et hübriidhaava kasvatamine lühikese raieringiga majandatava lehtpuuna Balti regioonis ei mõjuta märgatavalt saprofüütsete mulla mikroorganismide ohtrust ja mitmekesisust.

3.6. Impact of six-year-long organic cropping on soil microorganisms and crop disease suppressiveness

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Impact of six-year-long organic cropping on soil microorganisms and crop disease suppressiveness

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Abstract

The objective of the study was to conduct a complex investigation into microbial diversity in the soil, to determine the impact of six years' long organic agricultural practices on soil microbial communities after long-term conventional cropping. Classical microbiological methods were used for analysis of cultivable microorganisms, while molecular genetics methods targeted also uncultivable organisms. During the two year period significant fluctuations were observed in the total number of analyzed groups of cultivable soil microorganisms in particular fields and at particular sampling times. On average, significantly higher numbers of all groups of analyzed cultivable microorganisms were observed in organic agriculture fields in comparison to conventional fields, e.g., the number of bacteria had increased by 70%, actinobacteria by 290%, cultivable filamentous fungi by 110%, yeasts and maltose fermenting bacteria by 190%. Results obtained by molecular methods regarding fungal diversity and *Trichoderma* spp. DNA amount did not show such an increase. In contrast to the soil microbiological indicators, plant health, in terms of plant disease suppressiveness, had not improved – crop plants in organic agriculture fields were more severely or at the same level attacked by particular plant pathogenic fungi and bacteria.

Key words: organic and conventional agriculture, microbial diversity, ARDRA, qPCR, disease suppression.

Introduction

Agricultural management, such as crop rotation, tillage, compost, manure, herbicide and synthetic fertilizer application, and water regime, are key determinants of microbial community structure in soil. Vegetation is also an important factor since plants are providing microorganisms with specific carbon sources (Garbeva et al., 2004).

In an investigation in the semiarid Canadian prairie comparing annual legumes as green manure (green fallow) with tilled fallow-wheat and continuing wheat cultivation it was estimated that after six years of green fallow practices significant improvements were detected in several microbial characteristics such as colony counts of aerobic bacteria and filamentous fungi (Biederbeck et al., 2005).

Several investigations show long term positive influence of organic farming on soil quality and microbial activity in comparison with conventional farming, due to regular crop rotation, and absence of synthetic nutrients and pesticides (Shannon et al., 2002). The diversity of bacterial functional communities has been recorded to be higher in soils from organic farms, while species diversity was similar (Liu et al., 2007). Higher abundance and diversity of actinobacteria, important decomposers of organic material, is reported in organic tomato fields than conventional ones (Drinkwater et al., 1995). The ratios of Gram+ to Gram− bacteria and of bacteria to fungi have been reported to be higher in the fields with organic treatments than in the conventional treatments (Marschner et al., 2003).

However, investigations into soil fungal communities do not clearly indicate that they are always positively influenced by organic agriculture practices. In an investigation in southern Germany it was determined by the cultivation-independent approach (molecular methods), that fungal populations were almost entirely uninfluenced by the farming management practices. Whereas active population, investigated by the isolation of hyphae using a soil-washing technique and cultivation, showed a clear response to farming management practices (Hagn et al., 2003). The propagule numbers of *Trichoderma* has been shown to be higher in soils from conventional farms (Elmholt, Labouriau, 2005; Liu et al., 2007), but it depended on the year of analyses. In an investigation in Denmark it was determined that there were no significant differences of cultivable filamentous fungi (CFF) and yeasts among organically cultivated fields and fields with synthetic fertilizer and/or animal manure. There were differences only in the abundance of particular genera of CFF – *Penicillium* spp. and *Gliocladium roseum* were more represented under organic than conventional farming (Elmholt, Labouriau, 2005).

In order to estimate the impact of agricultural practices it is important to evaluate both soil microbial parameters, and disease suppressive capacity of the soil. For agricultural purposes it is important to reduce the level of soil-borne fungal and bacterial pathogens. Disease suppressive properties of the soil depend on various factors: soil texture, structure, pH, Ca content, agricultural practices (crop rotation, tillage, fertilizers and organic amendments), soil biota (microbial activity or soil respiration, microbial community diversity and composition, population size of particular microbial groups like actinobacteria) (Postma et al., 2008).

Only small part of soil fungi (17%) (Bridge, Spooner, 2001) and bacteria (0.1–1%) (Torsvik et al., 1996) are cultivable in laboratory conditions. Therefore nowadays two approaches are used to analyze soil microbial communities – conventional plating of cultivable microorganisms and molecular methods that are independent of cultivation. Amplified rRNA gene restriction analysis (ARDRA) gives genetic fingerprinting of communities, populations or phylogenetic groups. In soil microbiology this method is used to determine diversity within phylogenetic or functional groups of microorganisms (Lynch et al., 2004). Several studies have shown that quantitative polymerase chain reaction (PCR) can be used successfully to determine the abundance of specific groups of microorganisms in soil. An important genus of soil fungi analyzed with this method is *Trichoderma* that are known for their antagonistic activities against plant pathogens (Cordier et al., 2006).

The objective of our study was to conduct a complex investigation of microbial parameters in the soil of three organic and four conventional agriculture fields in order to estimate the impact of six-year-long organic agriculture practices in Northern temperate zone conditions, and to compare the characteristics of microbial populations with crop plant health and pathogen suppression. For the characterization of soil bacteria only classical microbiological methods that analyze cultivable bacteria were used, but soil fungal populations were assessed using both classical and molecular biology methods targeting also those organisms that are uncultivable under laboratory conditions. The hypothesis was that six years of organic agriculture practices after long-term conventional agriculture can show the changes in the conditions of soil microbial populations and/or plant health and pathogen suppression.

Materials and methods

Site description. Three fields of organic agriculture (Org1, Org2, Org3) and four fields of conventional agriculture (Conv4, Conv5, Conv6, Conv7) managed by State Priekuli Plant Breeding Institute, Latvia, were examined (Table 1). They are located ca. 123 m above sea level, 57°19′ N, 25°20′ E. All analyzed fields represented sod-podzolic soil (*Luvisol, LV*).

Fields of organic agriculture have been managed as described already for six years (since 2003). The green manure is applied in each field every six years (total nitrogen 120–200 kg ha^{-1}). Additionally the amelioration of soil is achieved by cultivating clover as the improvement through the nitrogen fixation, as well as by turning the plant residues into the soil. The crop rotation in organic fields is as follows: spring crops with clover, clover, winter crops, potatoes, spring crops and crucifers for green manure. In the conventional fields, winter crops and potatoes were grown sequentially. These fields have been under long-term conventional agriculture regime since 1912.

Other soil properties of fields and crops grown for the last three years are presented in Table 1. Humus, total nitrogen, phosphorus and potassium contents were determined by the State Plant Protection Service of Latvia in 2008 in organic fields and in 2007 in conventional fields. Fertilization regime of the conventional fields is given in Table 2, while applied pesticides are listed in Table 3.

Table 1. Crop rotation and soil properties

Field	Crop			Soil texture	Humus content %	Total N kg ha^{-1}	P_2O_5 kg ha^{-1}	K_2O kg ha^{-1}
	2007	2008	2009					
Org1	clover	potato	peas, rye[a]	loamy sand	2.3	28.12	161	150
Org2	potato	crucifers[b], oil radish[a]	barley	loamy sand	1.7	27.55	150	108
Org3	winter rye	winter rye	potato	loamy sand	1.5	25.66	99	104
Conv4	winter rye	potato	barley	loam	2.7	ND[c]	158	77
Conv5	clover	winter rye	potato	loam	2.3	ND[c]	184	126
Conv6	annual rye-grass	barley with vetch	beans	loamy sand	1.9	ND[c]	48	80
Conv7	oats	spring oilseed rape	barley	loam	2.3	ND[c]	115	102

[a] – green manure, [b] – mixture of oil radish, oil seed rape and mustard, [c] – not determined

Table 2. Fertilization of the conventional fields

Field	N:P:K in 2008	Amount kg ha^{-1}	N:P:K in 2009	Amount kg ha^{-1}
Conv4	11:9:20	500	6:15:30 additional N	350 70
Conv5	16:18:14 additional N	200 200	17:10:14	330
Conv6	without fertilization	0	without fertilization	0
Conv7	6:15:30 additional N surface fertilizing 18:18:18	100 100 5	without fertilization	0

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Table 3. Pesticides applied in the conventional fields

Field	Pesticides used in 2008	Amount per hectare	Pesticides used in 2009	Amount per hectare
Conv4	herbicide Zenkor fungicide Ridomil Gold fungicide Gloria	0.4 kg 2.5 kg 2.0 kg	herbicide Sekator	0.15 l
Conv5	herbicide Granstar Preiss	3.0 kg	herbicide Mistral insecticide Fastak herbicide Pantera fungicide Ridomil Gold fungicide Gloria fungicide Penkoceb	0.3 kg 0.2 kg 1.5 kg 2.0 kg 2.0 l 2 kg
Conv6	herbicide Bazagran herbicide Stomp	1 l 2 l	herbicide Bazagran herbicide Stomp	1 l 2 l
Conv7	herbicide Treflan herbicide Lontrel insecticide Fastak	4 l 0.3 l 0.4 l	herbicide Sekator	0.15 l

The total amount of precipitation 30 days before soil sampling was analyzed using raw data obtained from the data base of Latvian Environment, Geology and Meteorology Centre. Meteorological station in Priekuli was chosen for its proximity to the fields.

Soil sampling and estimation of pH and moisture content. Soil samples were taken in June and August 2008 and 2009. Nine subsamples were collected on a transect of each field at a depth of 10–15 cm (three subsamples in each corner of the field and three subsamples in the middle of the field, 100 g each). The subsamples were pooled together to create three larger samples for every field. Altogether 84 soil samples were analyzed. Samples were placed in sterile plastic bags "Whirl-Pak" ("Nasco", USA), stored at +4°C for a few days for the enumeration of cultivable microorganisms and after were stored at −20°C. The pH of the soil samples was measured in distilled water according to the ISO 10390 method. The moisture content of the soil samples was determined according to the ISO 11465 method.

Microbial parameters. In order to estimate the number of colony forming units (CFU) of cultivable microorganisms by a plate count method soil sample dilutions were prepared by adding 10 g of soil to 90 ml of sterile distilled water. Each soil sample was analyzed in two replicates. Suspensions were homogenized 1 h on a horizontal shaker. After that serial dilutions were prepared, and 0.1 ml of dilutions 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were used. Agarised malt extract ("Biolife", Italy) was used for cultivation of cultivable filamentous fungi (CFF), yeasts and particular groups of bacteria. The number of CFU was assessed after three days' incubation at 20 ± 2°C. The number of bacterial CFU was estimated on yeast extract peptone agar "Nutrient broth E" ("Int. Diagnostics Group", Great Britain). The number of CFU was assessed after three days' incubation at 20 ± 2°C. Number of CFU was expressed per gram of dry soil. Since actinobacteria represent an important group of soil bacteria, they were quantified separately.

Genera of filamentous fungi were determined after 10 days of incubation of the fungal CFU plates on malt extract agar according to morphological characteristics and light microscopy using keys (Barnett, 1957; Kiffer, Morelet, 2000).

In order to monitor plant health each growing season the information about the time of outbreak and severity of late blight (*Phytophthora infestans*), potato scab caused by *Streptomyces scabies* and black scurf of potato caused by *Rhizoctonia solani* was recorded.

Analyses of soil microbial diversity. DNA extraction. Total soil DNA was extracted using the PowerSoil™ DNA isolation kit ("MO BIO Laboratories", USA). Soil samples (250 mg) were homogenized using horizontal "Mixer Mill Type MM 301" ("Retsch", Germany) at a maximal speed of 30 Hz (1800 oscillations min^{-1}) for 10 minutes. DNA was extracted twice from each sample and all DNA extracts from the same field were pooled. The amount and purity of the DNA was determined spectrophotometrically using "Ultrospec 3100 Pro" ("Amersham Biosciences", Sweden) and by a 1% agarose 1 × TAE (w v^{-1}) gel electrophoresis.

Amplification with PCR. For ARDRA analyses of soil DNA the fungal nuclear ribosomal RNA region containing two internal transcribed spacers and the 5.8S rRNA gene (ITS1-5.8S-ITS2) was amplified with primers ITS1F and ITS4 (Gardes, Bruns, 1993) that are specific to the ITS1-5.8S-ITS2 region in *Ascomycota*, *Basidiomycota* and *Zygomycota* fungi. PCR reactions were carried out in 50 µl volume in an "Eppendorf Mastercycler Personal" ("Eppendorf", Germany). The reaction contained 2 u Hot Start *Taq* DNA polymerase, 5 µl 10X Hot Start PCR buffer, 0.2 mM dNTP, 2 mM $MgCl_2$, 0.75 µl bovine serum albumin 20 mg ml^{-1} (all reagents from "Fermentas", Lithuania), 0.5 µM of each primer ("Operon Biotechnologies", Germany), and 1 µl of DNA template. The PCR conditions were as follows: initial denaturation step of 4 min at 95°C, 40 s of denaturation at 95°C, 40 s of annealing at 52°C, 1 min of primer extension at 72°C (30 cycles) and final extension 10 min at 72°C.

After the PCR the amplification products were precipitated by addition of 450 µl of mixture containing 96% ethanol and sodium acetate (3 M, pH 5.0) in the ratio 19:1. The precipitated DNA was divided into two parts and digested with restriction endonuclease *Bsu*RI (Chabrerie et al., 2003) ("Fermentas", Lithuania). Restriction products were visualized in 6% polyacrylamide gel electrophoresis in two replicates. Gels were photographed with a "BioSpectrum AC Imaging System" and analyzed with software *Kodak 1D*. For the estimation of the Shannon-Weaver diversity index the following equation was used:

$H' = -\sum p_j \log_2 p_j$, where p_j = relative intensity of individual band (Gabor et al., 2003).

We calculated also the species richness that corresponds to the total number of distinct bands (number of operational taxonomic units, OTUs) (Gabor et al., 2003) in an ARDRA profile.

Quantitative real time PCR (qPCR). For the qPCR primers uTr and uTf specific to *Trichoderma* spp. were used (Hagn et al., 2007). Each soil sample was analyzed in three replicates. The reactions were carried out in 25 µl volume. The mixture contained 12.5 µl SYBR® Premix Ex Taq ("TaKaRa", Japan) for the year 2008 samples or Maxima™ SYBR Green qPCR Master Mix 2x ("Fermentas", Lithuania) for the year 2009 samples, 1 µM of each primer, 9.5 µl of sterile distilled water and 1 µl of the DNA template. The reaction conditions in "SmartCycler" ("Cepheid", USA) were the following: initial denaturation step of 60 s at 95°C, 40 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 60°C, 30 s of primer extension at 72°C. Serial dilutions of DNA from a pure culture of *Trichoderma harzianum* (MSCL 309) was used to construct the standard curve (separate for each SYBR Green kit).

Ribosomal RNA gene sequencing. For sequencing of the fungal ribosomal RNA gene region the genomic DNA of 39 isolates representing dominant filamentous fungi and sterile mycelia was amplified with primers ITS1F and ITS4, while representatives from genera *Penicillium* and *Aspergillus* were amplified also with modified fungal β-tubulin primers T12 (5'-TCT GGA TGT TGT TGG GAA TCC-3') and T22 (5'-AAC AAC TGG GCC AAG GGT CAC-3'), originally developed by O'Donnell and Gigelnik (1997). Both strands of the amplification products were sequenced with "BigDye Terminator v3.1 Cycle Sequencing Kit" ("Applied Biosystems", USA). Obtained sequences were analyzed using *Staden Package 1.6.0* release. The resulting consensus sequences were used in BLASTN homology search against the NCBI nucleotide database. Species were determined combining information from the most homolog sequence in the database and light microscopy results using keys (Barnett, 1957; Kiffer, Morelet, 2000).

Statistical analyses. Significance of differences between means was analyzed by the Tukey-Kramer test ($\alpha = 0.05$). Correlation analyses were done with *Excel* ("Microsoft", USA). Multiple regression analysis was done with *R package* (http://www.R-project.org) to evaluate the influence of several factors – soil moisture content, soil pH, precipitation, soil nutrient content and crop. In order to determine the impact of the crop on the microbial diversity, the crops were ranked as follows: 1) potatoes, 2) spring rape, 3) winter rye, 4) barley, 5) barley with vetch, 6) beans, 7) crucifers, 8) peas and rye (green manure).

Results and discussion

Soil chemical and physical parameters. The total amount of rainfall during the 30 days preceding the soil sampling was as follows: 83.80 mm in June 2008, 63.20 mm in August 2008, 20.60 mm in June 2009 and 141.50 mm in August 2009. Slight differences in soil moisture content were found at particular sampling times – 14.2 ± 2.9% in June 2008, 18.4 ± 1.2% in August 2008, 21.7 ± 1.2% in June 2009 and 14.3 ± 2.2% in August 2009.

Soil pH did not change significantly during the analyzed two year period – it ranged from 6.60 ± 0.41 to 6.82 ± 0.21 and it did not differ significantly between the organic and conventional cropping systems. Similarly, other parameters such as humus content, potassium and phosphorus content on average was similar in both groups of fields, the exceptions were fields Org3 and Conv6 with reduced level of phosphorus and fields Conv4 and Conv6 with reduced level of potassium (Table 1). The impact of various elements content on the analyzed microbial parameters is discussed later.

Soil microbial parameters. During the two year period, the level of cultivable bacteria was constant in the field Conv4, while in other fields marked changes were observed, e.g., substantial increase of bacterial CFU were detected in fields Org1 and Org2 in the August of 2009 (Fig. 1a). However, the results of Tukey-Kramer test showed that these differences were not statistically significant. Significant differences were found in the field Conv5 comparing both sampling years – in 2009 the number of bacterial CFU was increased 2.14 times ($F_{5, 5} = 11.16$, $P = 0.007$). The comparison of log CFU data showed that on average the total number of bacteria was significantly higher in organic agriculture fields in comparison with conventional fields ($F_{35, 47} = 6.23$, $P = 0.02$). The number of bacteria in organic fields exceeded that in conventional fields by 70%.

There was a tendency that at the end of the summer in year 2008 the number of actinobacteria in all fields decreased (except field Org2) but in year 2009 – in all fields increased (Fig. 1b) but these changes were not statistically significant. According to the Tukey-Kramer test the total number of actinobacteria was significantly higher in organic agriculture fields ($F_{35, 47} = 6.07$, $P = 0.02$) – on average almost four times if comparing results of two years.

Total number of yeasts and maltose utilizing bacteria was fluctuating during research period and on average it was higher in samples of the year 2009 (Fig. 1d) and also in organic agriculture fields in general in comparison to conventional fields – on average by 190% (statistically not significant).

The ratio of bacteria to fungi differed significantly in particular sampling times (Fig. 2). On average the ratio of bacteria to fungi was significantly higher in the conventional fields (498 *v.s.* 312; $F_{35, 47} = 5.29$, $P = 0.024$), which is in line with previously published investigation of old mining sites in Germany. In this investigation, higher ratio of bacteria to fungi determined by signature phospholipid fatty acids was detected in the fields with long term organic treatments (fertilization with manure, sewage sludge or straw) in comparison with conventional treatments (NPK mineral fertilizer treatment with all plant residues removed; mineral fertilizer treatment with plant residues incorporated into the soil). These results were explained by differences in organic matter composition and corresponding substrate availability among different treatments (Marschner et al., 2003).

A common tendency was observed that total number of CFF was increased in the year 2009 in all fields with exception of field Conv7 but these differences were statistically significant only in fields Org1 ($F_{5, 5} = 11.98$, $P = 0.006$) and Org2 ($F_{5, 5} = 21.39$, $P = 0.0009$). The explanation, why the total number of CFF increased significantly in the second year in almost all fields, is still lacking, since none of the factors included in the regression models explained this shift. In spite of the fact that field Conv5 received fungicides (mancozeb and others) several times during the second summer (Table 3) the total number of CFF was increased 9.5 times at the end of the August 2009 in comparison with previous level (Fig. 1c). Data about dominating CFF genera showed that

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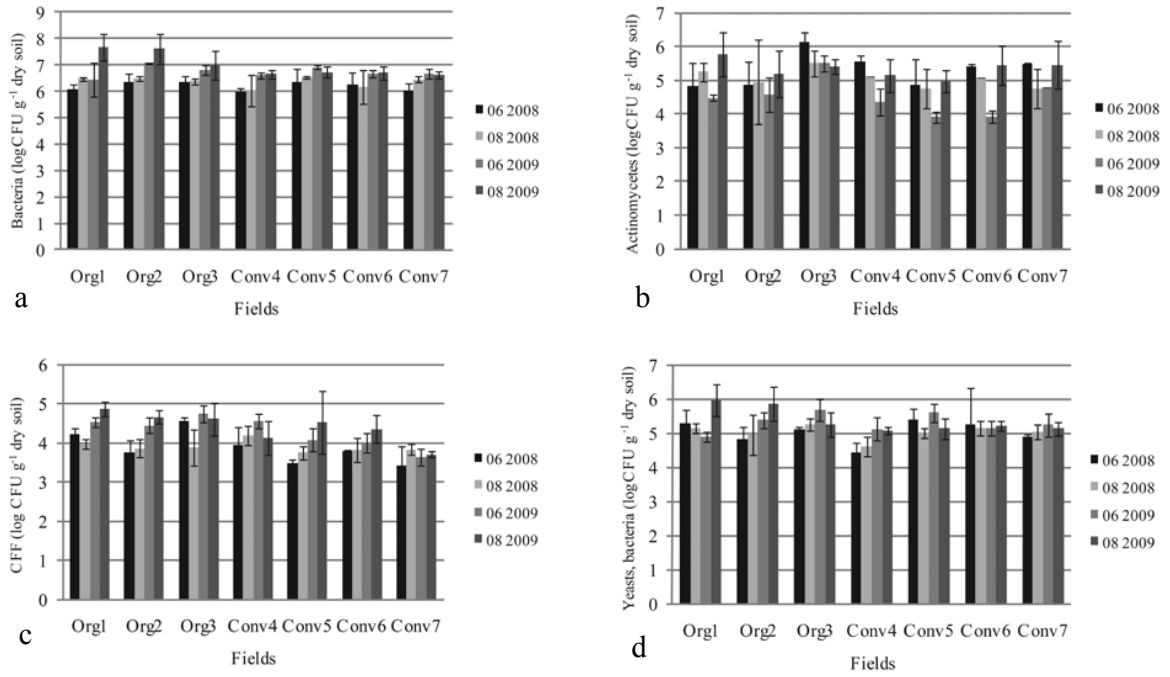

Figure 1. Total number of cultivable microorganisms (± S.D.) in each field at four sampling times (n = 6): a) total number of bacterial CFU, b) total number of CFU of actinobacteria, c) total number of cultivable filamentous fungi (CFF), d) total number of yeasts and maltose utilizing bacteria

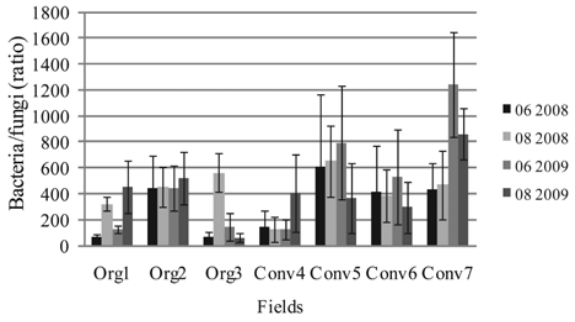

Figure 2. Ratio of bacteria to fungi (± S.D.) in each field at four sampling times (n = 6).

especially the number of CFU of *Mucor* spp. and sterile mycelia was increased in the year 2009, while CFU of other genera remained unchanged. It contradicts other investigations which found that application of such fungicide as mancozeb in amount 10 mg kg^{-1} soil decreased the amount of fungi for at least 3 months (Doneche et al., 1983), although the concentration of mancozeb applied on the Conv5 field was significantly lower (Table 3). In general, total number of CFF was significantly higher in organic fields ($F_{35, 47} = 1.40$, $P = 0.009$). The increase of CFF numbers in organic agriculture fields was on average approximately by 110%.

Changes in abundance of dominant fungal genera (*Trichoderma*, *Mucor*, *Mortierella*, *Penicillium* and *Verticillium*) and sterile mycelia (not sporulating after 10 days of incubation) were evaluated in the two year period. Similarly to the investigations of Liu et al. (2007), in our study there were no statistically significant differences in the propagule numbers of *Trichoderma* genus among fields of organic and conventional agriculture. It is assumed that *Trichoderma* spp. are less affected by a soil disturbance (after the use of pesticides) than other soil fungi, and are able quickly to colonize niches left by other organisms in conventional fields (Liu et al., 2007). The most abundant genus was *Penicillium* – on average 37.84 ± 14.35% of all fungi, while other genera were represented by 5–10% of all CFF and sterile mycelia covered 32.97 ± 10.12%. In organic fields only propagule numbers of *Penicillium* ($F_{11, 15} = 5.74$, $P = 0.02$) and *Verticillium* ($F_{11, 15} = 5.16$, $P = 0.03$) were significantly higher than in conventional fields ($p < 0.05$). Higher numbers of *Penicillium* in organic fields have been recorded in the work of Elmholt and Labouriau (2005). Other genera were evenly abundant in both groups of fields. Average numbers of the five mentioned genera and sterile mycelia are given in Figure 3.

In June 2008, representatives from such less abundant genera as *Absidia* (field Org3), *Cephalosporium* (Org2) and *Botrytis* (Org3) were detected in small numbers. In June 2009, *Geomyces* spp. were isolated from all fields (1.11 ± 0.56) x 10^3 g^{-1} of dry soil) and *Staphylotrichum* spp. from the fields Conv4 and Conv5 in small numbers. *Fusarium* spp. and *Acremonium* spp. were isolated from the samples of August 2009 (Org1, Org3, Conv7 and Org1, Org2, Org3, Conv5, Conv6, respectively).

Consequently, in our investigation we found that colony counts of all groups of cultivable microorganisms (bacteria, actinobacteria, yeasts and maltose utilizing bacteria and CFF) were significantly higher in organic agriculture fields after six years of organic cropping than in continuous conventional fields (Fig. 4). This is in line with the results of Biederbeck et al. (2005) in the semiarid Canadian prairie where numbers of bacteria and filamentous fungi were several times higher in fields with partial fallow and with annual legumes as green manure in comparison to fields with tilled fallow – wheat

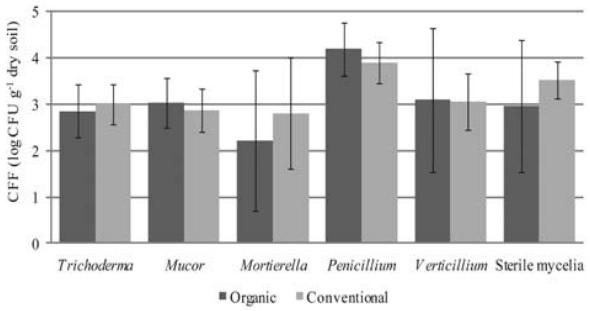

Figure 3. Average numbers (± S.D.) of the most abundant filamentous fungi genera and sterile mycelia (n = 72 for organic agriculture fields, n = 96 for conventional agriculture fields)

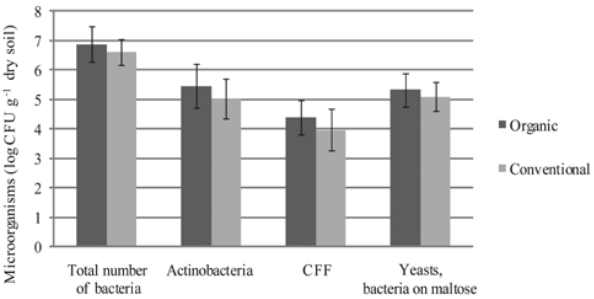

Figure 4. Average numbers (± S.D.) for two year period of all analyzed groups of cultivable microorganisms in organic and conventional fields (n = 72 for organic agriculture fields, n = 96 for conventional agriculture fields)

cropping system after six years of treatments. This investigation also proved that the microbiological attributes of the soil were sensitive and responsive to the beneficial influence of the particular cropping systems. Similarly, 1.6 times higher bacterial numbers under low input agriculture in comparison with high input agriculture have been recorded in an eight-week microplot experiment in the Netherlands (Bloem et al., 1992).

Organic agricultural practices are more favourable for soil microorganisms because they return more organic residues to the soil, and most of the N remaining in soil after the incorporation of green manure increases the mineralizable fraction of soil N. Such microbial population increases may be short-term or persist for at least one year after green manure incorporation (Biederbeck et al., 2005).

Soil microbial diversity. Examples of ARDRA results in 6% polyacrylamide gel electrophoresis are given in Figures 5a and 5b. Average results of the fungal diversity indices *H'* and number of OTUs are given in Figures 5c and 5d, respectively. There were no statistically significant differences among fields of organic and conventional agriculture, although the mean diversity index *H'* was higher in the organic fields in comparison to the conventional agriculture fields (2.56 *v.s.* 2.43). The numbers of OTUs obtained from the organic fields were also higher than from the conventional fields (7.71 *v.s.* 7.66). Similarly to our study, no significant differences were detected between the two agricultural regimes regarding number of phylotypes per field and Shannon diversity indices of arbuscular mycorrhizal fungi in onion fields in Netherlands using molecular methods (Galván et al., 2009).

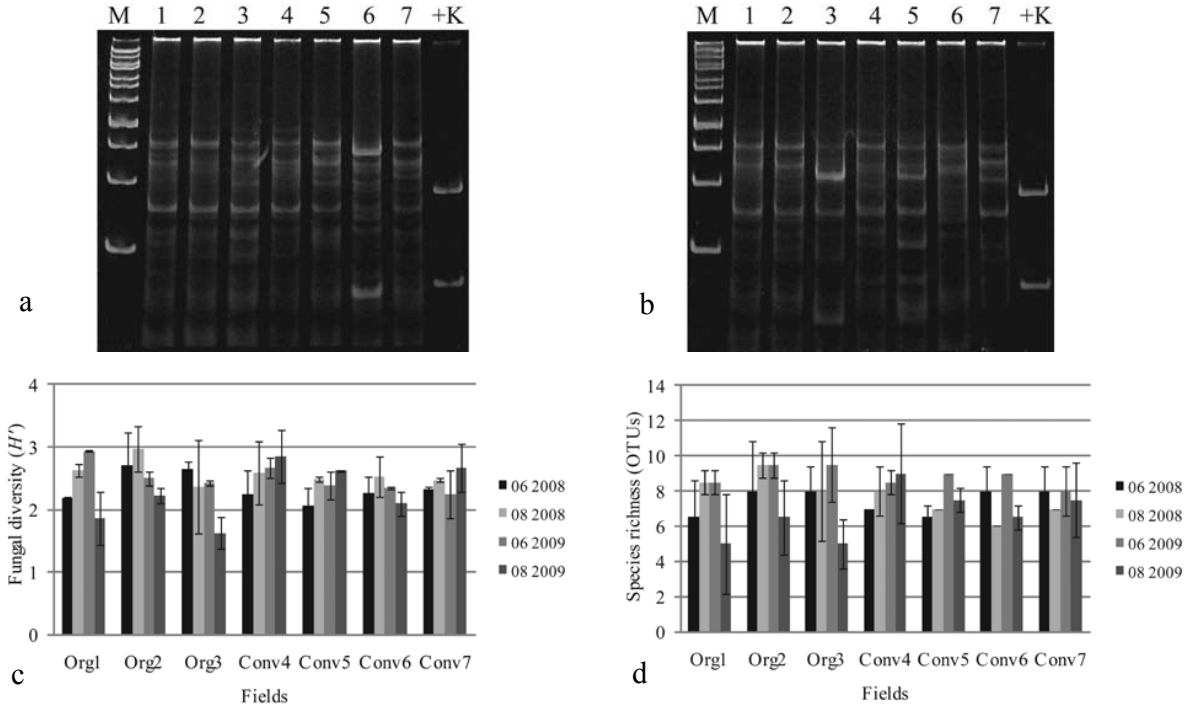

Figure 5. Results of ARDRA in 6% polyacrylamide gel electrophoresis: a) June 2008, lanes 1–3 organic agriculture fields, 4–7 conventional agriculture fields; b) August 2009, lanes 1–3 organic agriculture fields, 4–7 conventional agriculture fields; M – GeneRuler 1kb DNA Ladder ("Fermentas", Lithuania), +K – restriction results of *Trichoderma rossicum* DNA; c) Shannon-Weaver diversity index *H'* (± S.D.) of fungi in all analyzed fields at four sampling times (n = 2); d) number of OTUs (± S.D.) of fungi in all analyzed fields at four sampling times (n = 2)

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qPCR indicated an increase in the amount of *Trichoderma* spp. DNA in 2009, especially in August, in fields Org1, Org2, Org3, Conv6 and Conv7 (Fig. 6). However, there were no statistically significant differences among fields of organic and conventional agriculture, although the mean values of this parameter were higher in organic fields – 9.23 ng g^{-1} dry soil *v.s.* 7.17 ng g^{-1} dry soil.

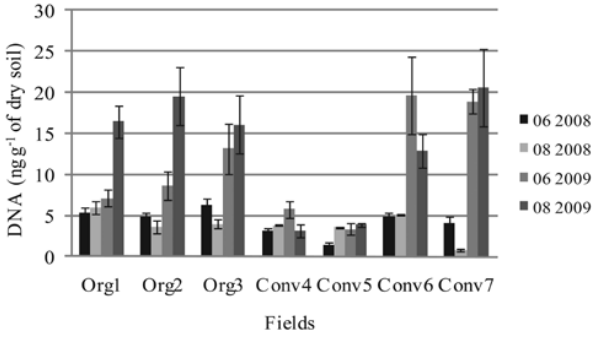

Figure 6. The amount of *Trichoderma* spp. DNA (± S.D.) in all analyzed fields at four sampling times (n = 3)

Significant part of all CFF was isolated as sterile mycelia. Sequencing showed that isolated sterile mycelia belonged to various fungal genera – *Bionectria*, *Stephanonectria*, *Arthrinium*, *Verticillium*, *Trichocladium*, *Phoma* and *Acremonium*. Two isolates remained unidentified also after sequencing. List of all sequenced fungal isolates is given in Table 4. One strain of each species was deposited in Microbial Strain Collection of Latvia (MSCL).

Sequencing enabled identification of fungal isolates to the species level with higher probability than according only to the morphological characteristics. Identified fungi mostly are saprophytic, but several plant pathogenic fungi were also detected, e.g., from the barley field (Org2) *Fusarium oxysporum* was isolated that causes root rot on barley as well as on other cereals and is common in Latvia (Treikale et al., 2008). From the field Conv4, where potatoes were grown in 2008, *Verticillium dahliae* was isolated that causes wilting in many plants and is recorded to be pathogenic also for potato (Kapsa, 2008). *Phoma eupyrena* that causes dry tuber rot of potato (Choiseul et al., 2007) was isolated from the field Conv5 planted with potato in 2009.

Aspergillus fumigatus, *Humicola grisea*, *Acremonium* sp., *Cladosporium cladosporioides*, *Mucor hiemalis*, *Penicillium canescens* and *Trichoderma hamatum* were isolated from those fields in our investigation, where rye has been cultivated within the last three years as crop or as green manure similarly to the study in Poland, where rye was used as a cover crop in tomato fields (Jamiołkowska, Wagner, 2005).

Results of statistical analyses. Multiple regression analyses showed that higher soil moisture content, for example, 21.7 ± 1.2% in June 2009, had significant negative impact on the total number of actinobacteria, while it had a positive effect on the number of sterile mycelia and species richness OTUs obtained by ARDRA method (Table 5). The total amount of precipitation in the 30 days preceding sampling significantly negatively affected the ratio of bacteria to fungi and the number of *Mortierella* spp.

According to the multiple regression analyses, the soil humus content (Table 1) had a negative impact on the total number of bacteria, total number of CFF and number of cultivable *Penicillium* spp. (Table 5). In the case of CFF, it contradicts the results of Hr elová et al. (1999), where the impact of the organic matter (oxidizable carbon) on soil microfungi was positive, but in the case of total number of bacteria and number of cultivable *Penicillium* spp. the observed impact was similar. Negative impact of humus content on soil fungi has been recorded in the work of Marschner et al. (2003).

Available phosphorus (P_2O_5) in the soil (Table 1) had positive impact on the total number of bacteria. In other investigations, positive impact of phosphorus was observed on soil saprophytic microfungi (Hršelová et al., 1999) but negative on species richness of arbuscular mycorrhizal fungi in soil macrocosms (Huang et al., 2005). Such contradiction can be explained by the fact that the numbers of phosphate solubilizing microorganisms vary from soil to soil (Gyaneshwar et al., 2002).

Available potassium (K_2O) in the soil (Table 1) had positive influence on the total number of CFF and yeasts and maltose utilizing bacteria. The nitrogen content in the fertilizer had negative impact on the total number of CFF but positive impact on the ratio of the bacteria to the CFF (Fig. 7). Negative impact of the phosphorus content in the fertilizer was observed on the total number of actinobacteria and on the amount of *Trichoderma* spp. DNA (data not shown). Positive impact of the potassium content in the fertilizer was observed on the total number CFF and on the fungal diversity estimated by ARDRA in agarose gels (data not shown).

Results of multiple regression analysis showed that soil pH did not affect significantly any of the analyzed microbial parameters. Several parameters (total numbers of CFU of *Trichoderma*, *Mucor*, *Verticillium*, sterile mycelia, and OTUs in agarose gels) were not affected by any of the chosen factors (not shown in Table 5).

From all factors that were different in both agricultural practices, only the use of artificial fertilizers was included in the regression models. Impact of such important factors as the use of pesticides and frequency of ploughing was not evaluated mathematically, although generally no negative effects of pesticides or ploughing intensity on the microbial numbers and diversity were observed. The frequency of ploughing depends not only on the chosen agricultural regime but also on the crop, and the crop according to the ranks applied in statistical analysis significantly affected only the total number of soil bacteria and in the case of potatoes – the CFU number of sterile mycelia. Crops that had potentially promotive effect on soil microorganisms (green manure, growth of legumes that increases the amount of nitrogen in the soil etc.), generally increased the number of bacteria.

Crop pathogens. In 2008, the first damage of the late blight (*Phytophthora infestans*) in organic fields was observed 7–10 days earlier than in conventional fields. Late blight significantly destroyed foliage (30–100%) in organic fields 10 to 14 days before it reached such level in conventional fields. In 2009, the first spots of the disease on potato leaves were observed at the same time on both environments, but significant foliage damage (5–100%) was assessed after 10 days in organic fields and only after 24 days in conventional fields. The application of fungicide delayed the late blight development in conventional fields and prolonged vegetation period for longer time. The late blight development was faster in 2008 than in 2009 due to more favourable weather conditions (more rainfall during August) in 2008. The rainfall in August 2009 was half of that for the same period in two previous years.

Table 4. Fungal isolates in organic and conventional fields

Field	Species	Amplified gene	Homolog sequence in NCBI	Max identity %	Strain in MSCL
Org1	*Penicillium canescens*	rDNA	FJ439586. 1	99	879
Org1	*Penicillium commune* *Penicillium crustosum*	rDNA β-tubulin	GQ458026. 1 FJ012877. 1	99 98	878
Org1	*Aspergillus fumigatus* *Aspergillus fumigatus*	rDNA β-tubulin	GQ461905.1 AY048754.1	99 99	870
Org1	***Bionectria ochroleuca***[a]	rDNA	AF106532.1	99	886
Org1	*Trichoderma rossicum*	rDNA	EU280089.1	99	883
Org2	*Penicillium griseofulvum* *Penicillium aurantiogriseum*	rDNA β-tubulin	DQ339570.1 FJ012878.1	99 95	874
Org2	Uncultured ascomycete[b]	rDNA	EU520630. 1	99	–
Org2	*Fusarium oxysporum*	rDNA	EU364863. 1	99	–
Org2	*Penicillium pinophilum*	rDNA	GU566216.1	98	880
Org2	***Bionectria ochroleuca***	rDNA	AF106532.1	99	–
Org2	*Penicillium canescens* *Penicillium crustosum*	rDNA β-tubulin	FJ439586.1 FJ012877.1	99 92	879
Org2	*Cladosporium cladosporioides*	rDNA	GQ458030.1	100	864
Org3	*Mucor hiemalis*	rDNA	EU326196.1	99	–
Org3	*Penicillium canescens* *Penicillium aurantiogriseum*	rDNA β-tubulin	FJ439586.1 FJ012878.1	99 91	875
Org3	***Stephanonectria keithii***	rDNA	EU273554.1	96	866
Org3	*Trichoderma hamatum*	rDNA	GQ220703.1	98	867
Org3	*Amylomyces rouxii*	rDNA	AY238888.1	99	865
Org3	*Cladosporium macrocarpum*	rDNA	EF679380.1	100	887
Org3	***Arthrinium sacchari***	rDNA	AF393679.2	99	881
Conv 4	*Trichoderma rossicum*	rDNA	EU280089.1	99	–
Conv 4	*Humicola grisea*	rDNA	AY706334.1	100	868
Conv 4	*Aspergillus fumigatus*	rDNA	AY214448.1	100	–
Conv 4	*Talaromyces ucrainicus*	rDNA	AY533694.1	99	885
Conv 4	*Penicillium aurantiogriseum* *Penicillium aurantiogriseum*	rDNA β-tubulin	AJ005488.1 FJ012878.1	100 99	–
Conv 4	***Verticillium dahliae***	rDNA	DQ282123.1	97	863
Conv 4	*Penicillium melanoconidium*	rDNA	AJ005483.1	100	871
Conv 4	***Trichocladium asperum*** Uncultured soil fungus	rDNA rDNA	AM292050.1 DQ420780.1	99 99	–
Conv 4	*Geomyces destructans*	rDNA	EU854572.1	98	884
Conv 4	*Paecilomyces marquandii*	rDNA	GU566261.1	98	–
Conv 5	***Phoma eupyrena***	rDNA	AJ890436.1	100	–
Conv 5	*Penicillium commune* *Penicillium allii*	rDNA rDNA	EU833216.1 AJ005484.1	99 99	–
Conv 5	***Acremonium* sp.**	rDNA	AJ890439.1	99	–
Conv 5	**Uncultured ascomycete**	rDNA	AY833028.1	96	–
Conv 5	**Uncultured soil fungus**	rDNA	EU826895.1	99	873
Conv 6	*Acremonium* sp.	rDNA	AJ890439.1	99	–
Conv 6	*Paecilomyces marquandii*	rDNA	GU566261.1	99	888
Conv 6	*Penicillium verruculosum*	rDNA	AF510496.1	97	882
Conv 6	*Paecilomyces carneus*	rDNA	EU553305.1	98	887
Conv 6	*Paecilomyces carneus*	rDNA	AB258369.1	99	872

[a] – isolates with names in bold were isolated as sterile mycelia, [b] – *Scopulariopsis* sp. according to microscopy results

The prevalence of potato scab caused by *Streptomyces scabies* and black scurf of potato caused by *Rhizoctonia solani* was similar in the fields of both agricultural practices.

Consequently, in contrast to the soil microbiological indicators that showed improvement after six years of organic cropping in comparison to the conventional agricultural fields, the plant health, in terms of plant disease suppression, was been improved. Controversial results about the capacity of minimum tillage and organic agriculture systems to reduce the disease levels, for example, of common root rot of cereals caused by *Cochliobolus sativus*, *Verticillium* wilt and common scab of potato, have been obtained in previous investigations (Bailey, Lazarovits, 2003). One of the reasons for heavier infection of potato with *Phytophthora infestans* in organic fields could be the fact that soil can act as a reservoir of the inoculum of pathogenic fungi, for example oospores of late blight *Phytophthora infestans* can survive in the soil in the absence of the host for several years (Drenth et al., 1995).

Fungal activity measured as fungal biomass has been proved to correlate with *R. solani* suppression in soil (Postma et al., 2008). Our investigation shows that increase in the number of CFF does not result in the disease suppression. One of the explanations is that six years is a too short time period to reduce the plant pathogen levels in the soil because the crop rotation has gone through the whole cycle only one time.

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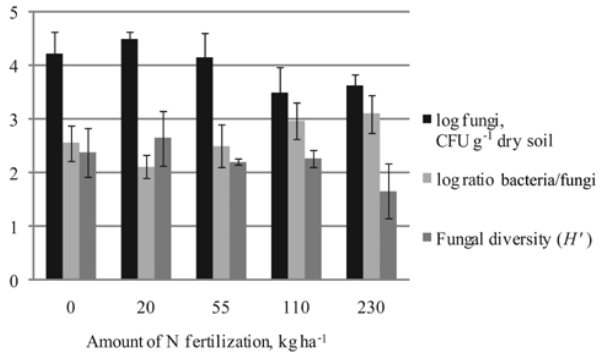
Table 5. Results of multiple regression analysis

Factor	CFF	*Mortierella*	Sterile mycelia	Yeasts and bacteria	Bacteria	Actinobacteria	Ratio bacteria to fungi	*Trichoderma* spp. DNA	*H'*	OTUs
Soil moisture	NS[a]	NS	0.02	NS	NS	<0.001[b]	NS	NS	0.005	<0.001
Total precipitation	NS	0.03[b]	NS	NS	NS	NS	0.03[b]	NS	NS	NS
Soil humus content	0.01[b]	NS	NS	NS	0.04[b]	NS	NS	NS	NS	NS
P_2O_5	NS	NS	NS	NS	0.006	NS	NS	NS	NS	NS
K_2O	0.003	NS	NS	0.005	NS	NS	NS	NS	NS	NS
N content in fertilizer	0.01[b]	NS	NS	NS	NS	NS	0.0009	NS	NS	NS
P content in fertilizer	NS	NS	NS	NS	NS	0.0007[b]	NS	0.005[b]	NS	NS
K content in fertilizer	0.002	NS	NS	NS	NS	NS	NS	NS	NS	NS
Crop	NS	NS	NS	NS	0.035	NS	NS	NS	NS	NS
Multiple R^2	0.51	0.18	0.19	0.33	0.37	0.51	0.43	0.27	0.27	0.47
p	0.002	0.03	0.02	0.007	0.01	0.0002	0.009	0.005	0.005	<0.001

[a] – not significant, [b] – negative impact



Note. Without nitrogen in the mineral fertilization – fields Org1, Org2, Org3, Conv6 in both years and Conv7 in 2009; 20 kg ha^{-1} – field Conv4 in 2009; 55 kg ha^{-1} – fields Conv4 in 2008 and Conv5 in 2009; 110 kg ha^{-1} – Conv7 in 2008; 230 kg ha^{-1} – Conv5 in 2008.

Figure 7. The impact of the nitrogen content in the mineral fertilizer on the total number of CFF, ratio of the bacteria to the CFF and fungal diversity index *H'* (± S.D.)

Conclusions

1. After six years of organic cropping practice, significantly higher numbers of all groups of cultivable microorganisms (bacteria, actinobacteria, yeasts and filamentous fungi) were observed in organic agriculture fields in comparison to conventional fields. In the case of filamentous fungi in organic fields propagule numbers of *Penicillium* and *Verticillium* were significantly higher. There were no statistically significant differences in the propagule numbers of *Trichoderma* and other dominant filamentous fungi genera (*Mucor*, *Mortierella*, *Absidia*, *Cephalosporium*, *Geomyces*, *Staphylotrichum*, *Fusarium*, *Acremonium*) among fields of organic and conventional agriculture.

2. Results obtained by molecular methods regarding fungal diversity and *Trichoderma* spp. DNA amount did not show any significant difference among fields of both agricultural managements although the tendency for organic fields to have higher values of both parameters was observed.

3. Soil filamentous fungi identified by microscopic and molecular methods were mostly saprophytic, but several plant pathogenic fungi were also detected from both groups of fields – *Fusarium oxysporum*, *Verticillium dahlia*, *Phoma eupyrena*.

4. The plant health, in terms of plant disease suppressiveness, had not improved – crop plants in organic agriculture fields were more severely attacked by *Phytophthora infestans*, and at the same level for *Streptomyces scabies* and *Rhizoctonia solani*.

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Ekologinės žemdirbystės taikymo (šešerius metus) įtaka dirvos mikroorganizmams ir augalų ligų slopinimui

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Santrauka

Tyrimų tikslas – kompleksiškai ištyrinėti dirvožemio mikrobų įvairovę ir nustatyti ekologinės žemdirbystės taikymo šešerius metus įtaką mikrobų bendrijoms po ilgalaikio tradicinės žemdirbystės taikymo. Analizuojant dirvožemio kultivuojamus mikroorganizmus taikyti klasikiniai mikrobiologiniai metodai, tiriant nekultivuojamus organizmus – molekulinės genetikos metodai. Per dvejų metų laikotarpį nustatyti ryškūs tyrinėtų kultivuojamų mikroorganizmų grupių bendro kiekio svyravimai tam tikruose laukuose tam tikrų pavyzdžių ėmimo metu. Visų analizuotų kultivuojamų mikroorganizmų grupių gerokai didesnis vidutinis kiekis buvo nustatytas ekologinės žemdirbystės laukuose, palyginti su tradicinės žemdirbystės laukais, pvz., bakterijų kiekis padidėjo 70 %, aktinobakterijų – 290 %, kultivuojamų siūlinių grybų – 110 %, mieles ir maltozę fermentuojančių bakterijų – 190 %. Grybų įvairovės ir *Trichoderma* spp. DNR kiekio tyrimų rezultatai, gauti taikant molekulinius metodus, tokio padidėjimo neparodė. Kitaip nei dirvožemio mikrobiologiniai rodikliai, augalų sveikata ligų slopinimo atžvilgiu nepagerėjo – ekologinės žemdirbystės laukuose augalai buvo labiau arba vienodai pažeisti patogeninių grybų ir bakterijų.

Reikšminiai žodžiai: ekologinė ir tradicinė žemdirbystė, mikrobų įvairovė, ARDRA, qPCR, ligų slopinimas.

4. Discussion

In all investigations only weak or medium strong correlations were observed between the results obtained with classical microbiology methods and molecular biology methods. This can be explained by the fact that the largest part of soil microorganisms is uncultivable under laboratory conditions. Nevertheless, both groups of methods gave important information about the soil microbial populations. In the investigation I correlation between the fungal diversity indexes obtained by conventional plating methods (H'_{CFF}) and by molecular methods (H'_{ARDRA}) was weak (Pearson's correlation coefficient r = 0.45). A stronger correlation of H'ARDRA was found with number of CFF (r = 0.50 – 0.61), total soil DNA amount (r = 0.54 – 0.72), and fungal DNA amount (r = 0.58). The correlation between the number of CFU of *Trichoderma* spp. and *Trichoderma* spp. DNA amount was weak – r = 0.20. It can be explained with the fact that not all species or strains of *Trichoderma* genus are sporulating under standard laboratory conditions (Ellison et al. 1981; Schrüfer and Lysek 1990) and they were counted as sterile mycelia. In the investigation II the correlation between the results of conventional plate count (number of CFF) and the amount of fungal DNA was weak (r = 0.18), and between H'_{CFF} and H'_{ARDRA} was even negative (r = –0.11). This observation may reflect the accumulation of unculturable or psychrophilic fungi that experience optimum growth conditions under the snow cover in presence of decaying plant litter as it was suggested in the investigation of temperate deciduous forest soil microbial communities that soil freezing creates new niches for certain microbial taxa (Aanderud et al. 2013). Their DNA could be amplified and identified, but we still could not grow corresponding fungi in standard laboratory conditions. In the investigation III the correlation between the results of conventional plate count method (number of CFF) and the amount of fungal DNA was weak (r = 0.26). A stronger correlation was found between number of CFF and H'_{ARDRA} (r = 0.44) and H'_{CFF} (r = 0.51). The average values of H'_{CFF} were 1.32 and those of H'_{ARDRA} – 2.44. In the investigation IV the correlation between H'_{CFF} and H'_{ARDRA} was weak – r = 0.19. In the investigation V medium strong correlations where obtained between the results of plate count method and H'_{ARDRA} – number of bacteria on TSA and bacterial diversity had Pearson's correlation coefficient r = 0.50, and in the case of number of yeasts and maltose using bacteria on MEA and fungal diversity the r was 0.69. The correlation between the values of H'_{CFF} and H'_{ARDRA} was negative. In the investigation VI H'_{CFF} values were not estimated but the correlation between the number of CFF and H'_{ARDRA} was near to zero that can be explained by the limited ability of plate count method to target only cultivable fungi.

In general (investigations I-V) there was a tendency that the abundance and diversity of microorganisms decreased with the soil depth (0 – 30 cm). The impact of sampling depth was statistically significant ($p < 0.05$). Other environmental factors (soil moisture content, average air temperature at

the sampling day, soil pH) had various impact on analyzed soil microbial variables depending from particular conditions (combination of mentioned factors, other factors such as soil type, microrelief) in each investigation or even in each sampling plot as it was observed in the monitoring of seasonal changes of spruce forest soil microbial populations.

Soil moisture content in the investigation I varied from 3 to 45%, and stepwise multiple regression analyses showed that it significantly positively affected the number of cultivable filamentous fungi on MEA ($p = 0.01$), the amount of fungal DNA ($p < 0.001$) and *Trichoderma* spp. DNA amount ($p = 0.01$). In the investigation II the soil moisture content varied from 3 to 76% and it significantly positively affected the number of filamentous fungi ($p = 0.0009$) and the total number of cultivable microorganisms ($p = 0.002$). Soil moisture content in the investigation III varied from 7 to 77% and significantly positively affected the number of filamentous fungi on MEA ($p < 0.001$), the total amount of soil DNA, the total amount of fungal DNA and the total amount of *Trichoderma* spp. DNA ($p < 0.001$). Soil moisture content in the investigation IV varying from 7 to 72% positively influenced yeasts and maltose-utilizing bacteria on MEA ($p < 0.0001$) but negatively the percentage of *Penicillium* spp. ($p = 0.03$) and the total number of fungal genera ($p = 0.04$). Soil moisture content in the investigation V that varied from 14 to 53% positively influenced almost all tested variables of soil microorganisms with the exception of the total number of bacteria on TSA and bacterial diversity (H') that were not significantly affected. Soil moisture content in the investigation VI varied from 9 to 23% and it significantly negatively affected the number of actinobacteria ($p < 0.001$) but positively the fungal diversity index H' ($p = 0.005$).

In two investigations (I and III) the soil moisture content positively affected *Trichoderma* spp. DNA amount, although the seasonality studies of *Trichoderma* species in spruce forest soil in Canada showed that members of this genus are more influenced by the competition among soil fungi than environmental factors such as soil moisture content (Widden and Abitbol 1980). This explains why this parameter was not affected by the soil moisture content in other investigations (II, VI) where it was assessed.

The general trend that almost all microbial variables tested in all investigation decreased by low soil moisture levels can be explained by the fact that in such conditions the diffusion processes of soluble substrates are limited and the mobility of microorganisms is reduced. Fungi in general are more tolerant to low water potential than bacteria (Voroney 2007). The capability of *Penicillium* spp. to tolerate low moisture content of the soil has been observed in other studies. For example, in the investigation where the ability of *Penicillium chrysogenum* was assessed to cause soft rot in the wood buried in soil (Hamed 2013). In the investigation where the ability of *Penicillium verrucosum* to grow in wheat grain was detected it was found out that the fungus was not able to grow at the moisture content less than 16% (Czaban et al. 2006). Majority of soil fungi are able to grow in the soil considerably drier than the permanent wilting point of higher plants (Griffin 1963).

In the investigation I the soil pH varied from 4.46 to 7.90 and it negatively affected the number of cultivable filamentous fungi on MEA ($p = 0.01$). In the investigation III where soil pH values ranged from 3.50 to 5.78 it significantly negatively affected yeasts and maltose-utilizing bacteria on MEA ($p = 0.02$) and the total number of microorganisms on MEA ($p = 0.005$). In the investigation IV

where pH values ranged from 3.72 – 5.08 the highest pH values negatively influenced the number of filamentous fungi genera (p = 0.008). Although the soil pH values were similar in all sampling plots of the investigation V (6.03 – 7.12), the regression models showed that soil pH significantly positively influenced the number of filamentous fungi on MEA (p = 0.002) and RBA (p = 0.02), and fungal (negatively, $p < 0.0001$) and bacterial diversity indices H' (positively, p = 0.02). In the soil of jack pine (*Pinus banksiana*) stands in Western Canada Staddon et al. (1998) found significant positive correlations between the functional diversity of microbial populations and soil pH estimated with sole-carbon-source utilization method. In general, fungi tolerate more acidic pH values. For example in the investigation where soil pH was changed artificially it was observed when soil pH changes from 8.3 to 4.5 fungal growth increases five times but bacterial growth decreases five times (Rousk et al. 2010).

Average air temperature of the sampling day in the investigation I varied from 4.0 to 22.7 °C and significantly positively influenced only number of cultivable filamentous fungi on MEA (p = 0.006). In the investigation II the air temperature of the sampling day varied from 6.4 to 17.0 °C significantly negatively influenced number of cultivable filamentous fungi on MEA (p = 0.045), but positively influenced the total number of microorganisms on MEA ($p < 0.0001$). In the investigation III the average air temperature of the sampling month varied from –10.7 to 17.9 °C and higher air temperatures positively affected the number of filamentous fungi on MEA (p = 0.01) and total amount of soil DNA (p = 0.02) but negatively influenced the total amount of fungal DNA ($p < 0.001$). Several fungal species isolated from the soil samples of the RP have been detected in arctic environments or recorded as growing at low temperatures, such as 0 °C: *G. vinaceus, G. pannorum, M. alpine* (Bergero et al. 1999); also *T. porosum* (Danielson and Davey 1973). The total amount of fungal DNA increased at lower air temperatures in both experimental plots in the investigation III that is in line with other investigations where it was determined that fungal populations dominate under the snow cover and that winter fungal populations are adapted to colder temperatures and more complex substrates (Lipson et al. 2002). Under the snow even when the air temperature is significantly below 0 °C unfrozen water remains around the soil particles (Schmidt and Lipson 2004).

In the investigation IV and VI sampling was done during summer. In the investigation V sampling was done from the end of the August till the beginning of the October but the impact of air temperature was not assessed.

Stepwise multiple regression analyses showed that elevated concentration of magnesium negatively affected the number of filamentous fungi on MEA in the investigation IV (p = 0.0002) where magnesium concentration varied from 0.19 ± 0.01 to 1.08 ± 0.073 g kg^{-1} and the number of filamentous fungi on RBA in the investigation V (p = 0.003) where magnesium concentration varied from 0.35 ± 0.02 to 3.98 ± 0.27 g kg^{-1}. Elevated concentration of magnesium negatively affected the number of represented fungal genera (p = 0.006) in the investigation IV and fungal diversity index H'_{ARDRA} in the investigation V (p = 0.001). In the investigation III the magnesium content positively influenced the number of yeasts and maltose utilizing bacteria on MEA, total number of microorganisms ($p < 0.001$) and *Trichoderma* spp. DNA amount (p = 0.02) but for the number of filamentous fungi it was not significant.

Positive impact of magnesium on organotrophic, oligotrophic and copiotrophic soil bacteria has

been observed in the study where soil was amended with 0.05 and 0.1 g kg^{-1} of Mg (Wyszkowska and Wyszkowski 2002) and on *Trichoderma* genus fungi (Duffy et al. 1997). A positive correlation between 0.1 and 0.2 g kg^{-1} of Mg in soil and termophylic microorganisms and *Trichoderma* spp. was detected also in the investigation of agricultural soils (Bulluck III et al. 2002). Negative correlations with available Mg content and vesicular-arbuscular mycorrhizal fungi spore abundance have been observed across a soil moisture–nutrient gradient (Anderson et al. 1984).

In its turn elevated concentrations of potassium positively affected the number of yeasts and maltose utilizing bacteria on MEA ($p < 0.05$) but negatively the percentage of *Mortierella* spp. ($p = 0.01$) in the investigation IV where potassium concentrations varied from 0.14 ± 0.01 to 0.80 ± 0.04 g kg^{-1}. In the investigation V where potassium concentrations varied from 0.40 ± 0.02 to 4.02 ± 0.18 g kg^{-1} elevated concentrations negatively influenced the total number of bacteria on TSA ($p = 0.0004$) but positively the number of filamentous fungi on RBA ($p = 0.01$). We can conclude that elevated concentrations of potassium positively affect soil filamentous fungi and yeasts.

The content of calcium that varied in the investigation IV from 0 to 4.54 ± 0.31 g kg^{-1} did not influence any of the analyzed soil microbial variables. In the investigation V the calcium content varied from 0.3 ± 0.02 to 71.32 ± 4.82 g kg^{-1} and it significantly positively affected the bacterial diversity index H'_{ARDRA} ($p < 0.0001$).

The phosphorus content that in the investigation IV varied from 0.02 ± 0.00 to 0.33 ± 0.02 g kg^{-1} and negatively influenced yeasts and maltose-utilizing bacteria on MEA ($p = 0.05$) but positively influenced the number of filamentous fungi on MEA ($p = 0.04$) and number of fungal genera ($p = 0.005$). In the investigation V where phosphorus content varied from 0.10 ± 0.02 to 0.36 ± 0.06 g kg^{-1} higher values of this element negatively affected the total number of bacteria on TSA ($p = 0.02$) and number of filamentous fungi on RBA ($p = 0.046$).

Although the total carbon and nitrogen content of the soil in the investigation IV and V had similar levels the C/N ratio values were significantly different. The C/N ratio values in the investigation IV ranged from 27 to 53 and negatively influenced the number of yeasts and maltose-utilizing bacteria ($p = 0.04$). The C/N ratio values in the investigation V ranged from 13 to 23, and higher values of this ratio correlated with lower values of several microbiological variables: the total number of bacteria on TSA ($p = 0.002$), the number of filamentous fungi on MEA ($p = 0.04$), the total amount of soil DNA ($p = 0.002$) and fungal diversity ($p = 0.02$). High C/N ratio positively influenced the number of filamentous fungi on RBA ($p = 0.002$). The nitrogen content in the investigation III varied from 0.06 to 0.63 % and it negatively affected the number of yeasts and maltose utilizing bacteria on MEA and total number of microorganisms ($p < 0.001$). In other investigations it is observed that nitrogen has positive impact on soil bacteria but negative impact on soil fungi (Högberg et al. 2007) that explains why the correlation obtained in the investigation III was negative – largest part from the total number of yeasts and maltose-utilizing bacteria on MEA is represented by yeasts because this microbiological medium is suitable only for a few bacterial groups.

The concentration of humic acids in the soil of aspen stands that ranged from 13.15 ± 0.01 to 77.30 ± 7.65 g kg^{-1} in the investigation V negatively influenced the number of yeasts, maltose utilizing bacteria ($p = 0.004$) and filamentous fungi on MEA ($p < 0.0001$), the number of yeasts ($p < 0.004$)

and fungi (p = 0.006) on RBA, and the total amount of soil DNA (p = 0.03). Similar trend to have smaller number of yeasts in the soil layers with more humus has been observed in the investigation of Priha et al. (2001) of soil microbial populations in pine, spruce and birch stands in Finland. In the investigation IV where concentration of humic acids in the soil of conifer stands was higher and varied from 17.5 ± 0.09 to 135.4 ± 23.76 g kg^{-1} it positively influenced filamentous fungi on MEA (p < 0.004) and the percentage of *Penicillium* spp. (p = 0.04). In its turn, the number of yeasts and maltose-utilizing bacteria was not significantly influenced by humic acid concentration. The humus content in the agricultural soil in the investigation VI varied from 1.5 – 2.7% and it negatively influenced the number of filamentous fungi on MEA (p = 0.01) and number of bacteria on nutrient agar (p = 0.04). From several studies on soil fungi it is known that litter-decomposing fungi are responsible for degradation and mineralization of soil organic matter but ascomycetes are related to modification and polymerization of humic substances, and also bacteria participate in the turnover of humic substances (Grinhut et al. 2007).

Land use type (forests, meadows, arable lands, former agricultural lands, organic and conventional agricultural practices) and vegetation (conifers, aspens, agricultural crops) have created differences in microbial populations not only in the terms of variation of soil bacterial and fungal abundance and diversity but also regarding species composition of soil saprophytic fungi, for example, from the soil of European aspen and hybrid aspen several fungal species were isolated that have been detected in other investigations of *Populus* and *Salix* spp. in Canada (Lumley et al. 2001) and Germany (Baum and Hrynkiewicz 2006) – *Acremonium strictum, Arthrinium sacchari, Cladosporium cladosporioides, Exophiala salmonis, Geomyces pannorum, Humicola fuscoatra, Hypocrea koningii, Mortierella alpina, Paecilomyces carneus, Penicillium janthinellum, Plectosphaerella cucumerina, Scytalidium lignicola, Trichoderma hamatum, Volutella ciliata, Wardomyces inflatus*.

Comparing 168 soil samples from forest stands infected with *Heterobasidion annosum s.l.*, 60 soil samples from healthy forest stands, 80 soil samples from European aspen stands, 80 soil samples from hybrid aspen stands and 84 soil samples from agricultural soils we can find genera and/or species of soil fungi that are typical for particular soils (Table 2). Common only in soil samples from forest stands infected with *Heterobasidion annosum s.l.* were such fungi as *Beauveria caledonica, Cylindrocephalum* sp., *Fomitopsis pinicola, Gilmaniella* sp., *Gloeophyllum sepiarium, Hypocrea citrina, Mortierella gamsii, Mortierella humilis, Neurospora* sp., *Rhizopus* sp., *Rhizosphaera kalkhoffii* and *Tolypocladium inflatum*. *F. pinicola* and *G. sepiarium* are brown rot fungi of boreal coniferous forest trees (Høiland and Bendiksen 1996). *H. citrina, M. gamsii* and *M. humilis* are plant growth promoting fungi (Eroshin and Dedyukhina 2002; Fakas et al. 2009; Haggag and Mohamed 2011). *B. caledonica* is a naturally occurring pathogen of forest beetles (Glare et al. 2008). *T. inflatum* is also an insect pathogen (Hodge et al. 1996). *R. kalkhoffii* is a needle pathogen of Norway spruce (Livsey and Barklund 1992). *Cylindrocephalum* sp., *Gilmaniella* sp., *Neurospora* sp. and *Rhizopus* sp. are common soil fungi (Barron 1964; Henderson 1965; Elad et al. 1981; Jacobson et al. 2004).

Abundant only in the soil of healthy forest stands were *Chalara longipes, Clonostachys candelabrum, Lophiostoma cynaroidis, Oidiodendron* sp., *Penicillium citreonigrum, Penicillium glaucoalbidum, Penicillium thomii, Phoma herbarum, Sagenomella* sp., *Stilbella byssiseda, Stilbella* sp., *Thanatephorus*

Table 2. Unique fungal species listed according to the ecological groups)

Soil of infected forests	Soil of healthy forests	Soil of hybrid aspen stands	Soil of European aspen stands	Agricultural soil
Saprophytes	**Saprophytes**	**Saprophytes**	**Saprophytes**	**Saprophytes**
Cylindrocephalum	*Lophiostoma cynaroidis*	*Emericella bicolor*	*Clonostachys divergens*	*Amylomyces rouxii*
Gilmaniella sp.	*Penicillium glaucoalbidum*	*Penicillium corylophilum*	*Emericella foeniculicola*	*Aspergillus fumigatus*
Neurospora sp.	*Penicillium janthinellum*	*Pseudallescheria fimeti*	*Hypocrea koningii*	*Cephalosporium* sp.
Rhizopus sp.	*Phoma herbarum*	*Scedosporium apiospermum*	*Lecanicillium kalimantanense*	*Penicillium allii*
Plant pathogens	*Sagenomella* sp.	**Plant pathogens**	*Leptospora rubella*	*Penicillium aurantiogriseum*
Rhizosphaera kalkhoffii	**Plant pathogens**	*Bionectria levigata*	*Mortierella clonocystis*	*Penicillium commune*
Plant growth promoting fungi	*Thanatephorus cucumeris*	*Botryotinia fuckeliana*	*Penicillium brasilianum*	*Penicillium griseofulvum*
Hypocrea citrina	**Decomposers of conifer litter**	*Nectria mariannaeae*	*Penicillium swiecickii*	*Penicillium pinophilum*
Mortierella gamsii	*Chalara longipes*	*Nectria vilior*	*Pyrenochaeta acicola*	*Penicillium verruculosum*
Mortierella humilis	*Clonostachys candelabrum*	*Neonectria radicicola*	*Sagenomella diversispora*	*Stephanonectria keithii*
Entomopathogens	*Penicillium citreonigrum*	*Neonectria ramulariae*	*Trichoderma tomentosum*	**Plant pathogens**
Beauveria caledonica	*Penicillium thomii*	**Plant root endophytes**	*Wardomyces inflatus*	*Penicillium melanoconidium*
Tolypocladium inflatum	**Entomopathogens**	*Aporospora terricola*	**Plant pathogens**	*Phoma eupyrena*
Brown rot fungi	*Tolypocladium cylindrosporum*	*Pyrenochaeta inflorescentiae*	*Gibberella pulicaris*	
Fomitopsis pinicola	**Mycorrhizal fungi**	*Rhizopycnis vagum*	*Common in wood*	
Gloeophyllum sepiarium	*Oidiodendron* sp.	*Tetracladium setigerum*	*Hyalodendriella betulae*	
	Mycopathogens	*Keratinophilic fungi*	*Ilyonectria rufa*	
	Stilbella byssiseda	*Eremomyces langeronii*	**Entomopathogens**	
	Stilbella sp.	**Plant growth promoting fungi**	*Isaria fumosorosea*	
		Gliomastix murorum	*Kernia pachypleura*	
		Hypocrea lixii	**Plant root endophytes**	
		Mortierella globulifera	*Leucostoma persoonii*	
		Mycorrhizal fungi	*Nectria lugdunensis*	
		Cadophora finlandica	*Keratinophilic fungus*	
		Mycopathogens	*Auxarthron umbrinum*	
		Paraconiothyrium sporulosum		
		Pathogens of nematodes		
		Plectosphaerella cucumerina		

cucumeris, Tolypocladium cylindrosporum. C. longipes, C. candelabrum, P. citreonigrum and *P. thomii* are decomposers of conifer litter (Brandsberg 1969; Koukol et al. 2004; Osono et al. 2006). *L. cynaroidis* is a saprophytic soil fungus (Curlevski et al. 2010). Species from the genus *Oidiodendron* form ericoid mycorrhiza in the roots of plants from *Vaccinium* genus (Dalpe 1986). *P. herbarum* is a saprophytic soil fungus with bioherbicide activity (Stewart-Wade and Boland 2004). *Sagenomella* spp. are common soil fungi in pine forest soil (Bååth 1981). The species *Stilbella byssiseda* has been detected in the soil of *Salix herbacea* in Austrian Central Alps (Oberkofler and Peintner 2008) and this fungus is known to colonize the fructifications of myxomycetes (Rogerson and Stephenson 1993). *T. cucumeris* (former *Rhizoctonia solani*) is a plant pathogen (Camporota and Perrin 1998). Entomopathogenic species *T. cylindrosporum* (synonym *Beauveria cylindrospora*) has been isolated from the soil under spruce colonized with Ips typographus in Czech Republic (Landa et al. 2001).

Represented only in the soil of hybrid aspen stands were following species: *Aporospora terricola, Bionectria levigata, Botryotinia fuckeliana* (anamorph *Botrytis cinerea*), *Cadophora finlandica, Emericella bicolor* (anamorph *Aspergillus bicolor*), *Eremomyces langeronii* (anamorph *Arthrographis kalrae*), *Gliomastix murorum, Hypocrea lixii, Mortierella globulifera, Nectria mariannaeae, Nectria vilior, Neonectria radicicola, Neonectria ramulariae, Paraconiothyrium sporulosum, Penicillium corylophilum, Plectosphaerella cucumerina, Pseudallescheria fimeti, Pyrenochaeta inflorescentiae, Rhizopycnis vagum, Scedosporium apiospermum, Tetracladium setigerum. A. terricola, R. vagum* and *T. setigerum* are plant root endophytes (Girlanda et al. 2002; Macia-Vicente et al. 2008; Sati et al. 2008). *Neonectria* spp. and *Bionectria* spp. formerly belonged to *Nectria* that are common as pathogens on various hardwood trees. *N. radicicola* occurs in the soil of root zone of many plants (Mantiri et al. 2001). *C. finlandica* is a member of ectomycorrhizal and ericoid mycorrhizal fungal association (Vrålstad et al. 2002). *B. fuckeliana* is a common plant pathogen (Faretra et al. 1988). *E. langeronii* is a keratinophilic fungus (Gené et al. 1996). *G. murorum, H. lixii, M. globulifera* are plant growth promoting fungi (Eroshin and Dedyukhina 2002; Khan et al. 2009; Fakas et al. 2009). *P. sporulosum* is common in soil and parasite on other fungi (Damm et al. 2008). *P. corylophilum* is a decomposer of forest litter (Osono et al. 2006). *P. cucumerina* is a pathogen of soil nematodes (Atkins et al., 2003). In temperate climates *Scedosporium* spp. and *Pseudallescheria* spp. can be isolated from soils with increased nitrogen concentrations and lowered pH (Kaltseis et al. 2009). *P. inflorescentiae* is an endophyte of *Taxus globosa* (Soca-Chafre et al. 2011).

Common only in the stands of European aspen were *Auxarthron umbrinum, Clonostachys divergens* (teleomorph *Bionectria* sp.), *Emericella foeniculicola* (anamorph *Aspergillus foeniculicola*), *Gibberella pulicaris, Hyalodendriella betulae, Hypocrea koningii, Ilyonectria rufa, Isaria fumosorosea, Kernia pachypleura, Lecanicillium kalimantanense, Leptospora rubella, Leucostoma persoonii, Mortierella clonocystis, Nectria lugdunensis, Penicillium brasilianum, Penicillium swiecickii, Pyrenochaeta acicola, Sagenomella diversispora, Trichoderma tomentosum, Wardomyces inflatus. A. umbrinum* is a keratinophilic fungus (Böhme and Ziegler 1969). *E. foeniculicola* is common soil fungus (Matsuzawa et al. 2012). *G. pulicaris* is a cosmopolitan soil saprophyte and pathogen of various plants (Desjardins et al. 1992). *H. betulae* has been found on trambling aspen logs (Kebli et al. 2012). *I. rufa* has been isolated from various coniferous trees (Cabral et al., 2012). *I. fumosorosea* and *L. kalimantanense* are

entomopathogenic fungi (Sukarno et al. 2009; Ayala-Zermeño et al. 2011). *L. persoonii* is an endophytic fungus of *Populus tremula* (Santamaría and Diez 2005). *N. lugdunensis* is an endophyte isolated also from the soil of transgenic poplar plantations (Danielsen 2012). *S. diversispora* has been isolated from the soil of aspen stand (Samson et al. 2011). *W. inflatus* has been isolated from the oak and spruce litter (Chigineva et al. 2009).

Common only in the agricultural soil were *Amylomyces rouxii* (*Rhizopus arrhizus*), *Aspergillus fumigatus, Cephalosporium* sp., *Penicillium aurantiogriseum, Penicillium commune, Penicillium griseofulvum, Penicillium melanoconidium, Penicillium pinophilum, Penicillium verruculosum, Phoma eupyrena, Stephanonectria keithii* (former *Nectria keithii*). *A. fumigatus* is often found on degrading vegetative material (Millner et al. 1977). *P. griseofulvum* has phosphate sulubilizing activity (Wakelin et al. 2004). *P. melanoconidium* has been isolated from various agricultural crop plants (Samson et al. 2004). *P. pinophilum* and *P. verruculosum* has been isolated from temperate agricultural soils (Ahmad and Malloch 1995; Chaineau et al. 1999). *P. eupyrena* causes dry tuber rot of potato (Choiseul et al. 2007).

Only two fungal species were represented in all land use groups, *Penicillium canescens* and *Paecilomyces carneus*. It is known that *P. canescens* degrades cellulose, chitin (Baldrian et al. 2011) and xylan (Chavez et al. 2006). *P. carneus* is decomposer of chitin (Gray and Baxby 1968) and entomopathogenic fungus (Sevim et al. 2010).

Presented only in agricultural soils and former agricultural soils with aspen stands were the following fungal species: *Acremonium felinum, Acremonium strictum, Bionectria ochroleuca, Geomyces destructans, Paecilomyces marquandii, Trichoderma rossicum, Verticillium dahliae*. Members from the genus *Acremonium* are known as decomposers of cellulose, carboxymethylcellulose and xylan (Ryckeboer et al. 2003). *B. ochroleuca* (anamorph *Clonostachys rosea*) has been isolated from the bark of *Salix* sp. (Hirooka and Kobayashi 2007) and has been used in the biocontrol of *Botrytis cinerea* (Cota et al. 2009) and it is colonizing plant roots and other parts as an endophyte (Chatterton et al. 2008). The genus *Geomyces* is common in the soil of colder climates (Minnis and Lindner 2013). *P. marquandii* has been described as entomopathogenic species (Sevim et al. 2010). *T. rossicum* has been isolated from the soil and rhizosphere of poplar and willow stands and as well as in *Hordeum* field in Austria (Wuczkowski et al. 2003). *V. dahliae* is a plant pathogen (Kapsa 2008).

Presented only in forest soils were the following species: A*spergillus cervinus, Beauveria bassiana, Penicillium spinulosum, Pochonia bulbillosa, Trichoderma asperellum, Trichoderma viride. A. cervinus* and *P. spinulosum* have been isolated from the forest soil in other investigations and they are characterized as degraders of plant cell-wall polysaccharides and organic phosphorus substrates (Daynes et al. 2008). *B. bassiana* is an entomopathogenic and plant endophytic fungus (Lygis et al. 2004; Brownbridge et al. 2012). *P. bulbillosa* has been isolated from bogs and can degrade spruce wood chips and *Sphagnum fuscum* substrate (Thormann et al. 2004; Thormann 2006). *T. asperellum* and *T. viride* have been isolated from the rhizosphere of *Pinus radiata* seedlings and roots of Norway spruce (*Picea abies*) (Holdenrieder and Sieber 1992; Hohmann et al. 2011).

Several fungal ecological groups are described – litter decomposing fungi, wood-attacking fungi, chitinolytic fungi, keratinophilic fungi, fungicolous fungi, coprophilous fungi, pyrophilous fungi,

ammonia fungi (Suzuki 2009), plant pathogens, plant endophytes and saprophytes (Rodriguez and Redman 1997). The lowest number of fungal ecological groups (five) were detected in agricultural soils – saprophytes, plant pathogens, plant root endophytes, plant growth promoting fungi and entomopathogenic fungi. In the soil of hybrid aspen and European aspen stands 11 ecological groups were presented, and additionally to the agricultural soil they were keratinophilic fungi, mycorrhizal fungi, mycopathogens, pathogens of nematodes, fungi common in wood and coprophilous fungi. In the soil of conifer forests fungi from 10 ecological groups were presented. Unique to these soils in comparison to other tested soils were decomposers of conifer litter and brown rot fungi. In common to forest soils and aspen stands were such ecological groups as mycorrhizal fungi, mycopathogens and coprophilous fungi but the particular species were partly different.

Although the ecological diversity was the lowest in agricultural soils, the genetical diversity estimated with ARDRA results in native 6% polyacrylamide gel electrophoresis was the highest - the average H'_{ARDRA} value was 2.38, and it was statistically significantly different from the average genetical diversity in the soil of aspen stands (2.04; $p = 0.004$) and conifer stands (1.91; $p = 0.0003$). The quantification of cultivable filamentous fungi using plate count technique showed than on average higher numbers of CFF were obtained from forest soils (4.70 log CFU g^{-1} soil) and soils of aspen stands (4.65 log CFU g^{-1} soil) but significantly lower numbers of CFF were obtained from agricultural soils (4.14 log CFU g^{-1} soil; $p < 0.0001$). Such controversial results about fungal populations in different land use groups can be explained by the fact that the largest part of soil fungi are not cultivable and that the soil community in agricultural soils contains large diversity of these fungi. In an investigation about soil fungal populations across different land use types in Carolina (United States) it was detected that arable land contained higher percentage of OTUs of such fungal taxa as Sordariomycetes and Leotiomycetes in comparison to loblolly pine (*Pinus taeda*) forest soil (Lauber et al. 2008).

The Sørensen's community similarity index C_s showed that the fungal populations were the most similar the more alike the soil groups were. For example, the highest C_s values were obtained comparing fungal populations of healthy forests with infected forests – 0.44 (Table 3). The most dissimilar fungal populations were between forest soils and agricultural soils where the index C_s was only 0.14.

Table 3. Total number of fungal species, number of shared species and values of Sørensen's community similarity indices C_s among all soil groups

Soil group (total number of species)	Soil of healthy forests (41)	Soil of hybrid aspen stands (55)	Soil of European aspen stands (68)	Agricultural soil (33)
Soil of infected forests (50)	$C_s = 0.44$ (20 shared species)	$C_s = 0.23$ (12 shared species)	$C_s = 0.29$ (17 shared species)	$C_s = 0.14$ (6 shared species)
Soil of healthy forests (41)	-	$C_s = 0.21$ (10 shared species)	$C_s = 0.16$ (9 shared species)	$C_s = 0.14$ (5 shared species)
Soil of hybrid aspen stands (55)	-	-	$C_s = 0.34$ (21 shared species)	$C_s = 0.27$ (12 shared species)
Soil of European aspen stands (68)	-	-	-	$C_s = 0.20$ (10 shared species)

If we compare healthy conifer stands with stands infected with *Heterobasidion annosum s.l.* it was observed that sampling plots with semihydromorphic soils including infected stands lack members of *Mortierella* genus or have them in low percentage but have increased percentage of members of *Umbelopsis* genus. Semihydromorphic soils have decreased percentage of CFU of *Penicillium* spp. and almost no *Trichoderma* spp. *Mortierella alpina* and *M. minutissima* have been isolated from serially washed healthy fine roots of Norway spruce (*Picea abies*) (Holdenrieder and Sieber 1992), but *Umbelopsis* spp. prefer soils with high nitrogen content (Jirout et al. 2011). Sampling plots with semihydromorphic soils in the upper soil layer (0 – 10 cm) had significantly higher amount of total nitrogen – 15.77 *vs.* 9.55 g kg^{-1} ($p < 0.05$).

In antagonism tests the isolates of *Penicillium* spp. in general reduced the growth rate of *H. annosum s.l.* but the antagonistic effect was not as strong as in other investigations in the case of *Trichoderma* spp. (Nikolajeva et al. 2012).

After six years of organic cropping practice, significantly higher numbers of all groups of cultivable microorganisms (bacteria, actinobacteria, yeasts and filamentous fungi, especially members of *Penicillium* and *Verticillium* genera) were observed in organic agriculture fields in comparison to conventional fields. There were no statistically significant differences in the propagule numbers of *Trichoderma* and other dominant filamentous fungi genera. The contradictory and not stable impact of agricultural practices on abundance of *Trichoderma* spp. has been observed in previous investigations as well (Elmholt et al. 2005; Liu et al. 2007). Results obtained by molecular methods regarding fungal diversity and *Trichoderma* spp. DNA amount did not show any significant difference among fields of both agricultural managements although the tendency for organic fields to have higher values of both parameters was observed. The plant health, in terms of plant disease suppressiveness, was not improved – crop plants in organic agriculture fields were more severely attacked by *Phytophthora infestans*, and at the same level for *Streptomyces scabies* and *Rhizoctonia solani*. Controversial results about the capacity of low tillage and organic agriculture systems to reduce the disease levels, for example, of common root rot of cereals caused by *Cochliobolus sativus, Verticillium* wilt and common scab of potato, have been obtained in previous investigations (Bailey et al. 2003). One of the reasons for heavier infection of potato with *Phytophthora infestans* in organic fields could be the fact that soil can act as a reservoir of the inoculum of pathogenic fungi, for example oospores of late blight *Phytophthora infestans* can survive in the soil in the absence of the host for several years (Drenth et al. 1995).

5. Conclusions

1. In all investigations only weak or medium strong correlations were observed between the results obtained with classical microbiology methods and molecular biology methods. This can be explained by the fact that the largest part of soil microorganisms is uncultivable under laboratory conditions. Both groups of methods gave important information about the soil microbial populations.
2. In general, there was a tendency that the number and diversity of microorganisms decreased with the soil depth (0 – 40 cm).
3. Other environmental factors (soil moisture content, average air temperature at the sampling day, soil pH) had various impact on analyzed soil microbial variables depending on particular conditions (combination of mentioned factors, other factors such as soil type, microrelief, microbiological media used for analyzes) in each investigation or even in each sampling plot as it was observed in the monitoring of seasonal changes of spruce forest soil microbial populations.
4. Land use type (forests, meadows, arable lands, former agricultural lands, organic and conventional agricultural practices) and vegetation (conifers, aspens, agricultural crops) have created differences in microbial populations not only in the terms of variation of soil bacterial and fungal abundance and diversity but also regarding the species composition of soil saprophytic fungi, for example, from the soil of European aspen and hybrid aspen several fungal species were isolated that have been detected in other investigations of *Populus* and *Salix* spp. in Canada and Germany.
5. Only two fungal species were represented in all land use groups – *Penicillium canescens* and *Paecilomyces carneus*, and they are saprophytic and entomopathogenic fungi.
6. The lowest number of fungal ecological groups (five) was detected in agricultural soils – saprophytes, plant pathogens, plant root endophytes, plant growth promoting fungi and entomopathogenic fungi. In the soil of hybrid aspen and European aspen stands 11 ecological groups were presented, and additionally to the agricultural soil they were keratinophilic fungi, mycorrhizal fungi, mycopathogens, pathogens of nematodes, fungi common in wood and coprophilous fungi. In the soil of conifer forests fungi from 10 ecological groups were presented. Unique to these soils in comparison with other tested soils were decomposers of conifer litter and brown rot fungi. Common also with aspen stands were such ecological groups as mycorrhizal fungi, mycopathogens and coprophilous fungi but the particular species were partly different.
7. The general trend was observed that almost all microbial variables tested in all investigations decreased by low soil moisture levels. The exception was the percentage of *Penicillium* spp., total number of fungal genera in coniferous forest soils, total number of bacteria on tryptic soy agar in the soil of aspen stands and number of actinobacteria in agricultural soils.
8. Soil pH in the investigations of coniferous forest soil varied from 3.5 to 5.1, in aspen soils and agricultural soils from 6.0 to 7.4. In general, higher values of pH negatively influenced all parameters related to soil fungi. Bacterial populations were less affected.

9. The average air temperature varied from –10.7 to +22.7 °C. Higher air temperatures controversially affected the number of filamentous fungi and positively influenced total amount of soil DNA but negatively influenced the total amount of fungal DNA. Several fungal species isolated from the soil samples during rest period have been detected in arctic environments or recorded as growing at low temperatures, such as 0 °C – *Geomyces vinaceus, Geomyces pannorum, Mortierella alpina* and *Trichosporon porosum*.
10. Soil microorganisms were significantly affected by the concentration of magnesium, potassium, phosphorus, nitrogen in the soil and C/N ratio.
11. Various response of soil microorganisms to high concentration of humic acids was observed in different soil groups – in forest soils populations of cultivable filamentous fungi were more adapted to higher humic acid concentrations in comparison with the agricultural soils and former agricultural soils with aspen stands where populations of cultivable filamentous fungi were presented in lower numbers.
12. If we compare healthy conifer stands with stands infected with *Heterobasidion annosum s.l.* it was observed that sampling plots with semihydromorphic soils including infected stands lack members of *Mortierella* genus or have them in low percentage but have increased proportion of members of *Umbelopsis* genus. Semihydromorphic soils have decreased percentage of CFU of *Penicillium* spp. and almost no *Trichoderma* spp. In antagonism tests the isolates of *Penicillium* spp. in general reduced the growth rate of *H. annosum s.l.* but the antagonistic effect was not as strong as in other investigations in the case of *Trichoderma* spp.
13. After six years of organic cropping practice, significantly higher numbers of all groups of cultivable microorganisms (bacteria, among them actinobacteria, yeasts and filamentous fungi, especially members of *Penicillium* and *Verticillium* genera) were observed in organic agriculture fields in comparison with conventional fields. There were no statistically significant differences in the propagule numbers of *Trichoderma* and other dominant filamentous fungi genera. Results obtained by molecular methods regarding the fungal diversity and *Trichoderma* spp. DNA amount did not show any significant difference among fields of both agricultural managements although the tendency for organic fields to have higher values of both parameters was observed. The plant health, in terms of plant disease suppressiveness, was not improved – crop plants in organic agriculture fields were more severely attacked by *Phytophthora infestans*, and at the same level for *Streptomyces scabies* and *Rhizoctonia solani*.

6. Defence theses

1. Due to the fact that the largest part of soil microorganisms is uncultivable under laboratory conditions, methods of classical and molecular microbiology show only weak or medium strong mutual correlation, and therefore the ecological diversity of cultivable soil filamentous fungi in agricultural soils is the lowest but the genetical diversity soil fungal population is the highest in comparison to former agricultural soils with aspen stands and conifer forest soils.
2. In respect to soil sampling strategy it should be taken into the account that number and diversity of microorganisms decreases with the soil depth and other environmental factors (soil moisture content, average air temperature at the sampling day, soil pH) have various impacts on analyzed soil microbial variables depending on specific conditions (combination of mentioned factors, other factors such as soil type, microrelief) in each investigation or even in each sampling plot.
3. Land use type and vegetation (forests, meadows, arable lands, former agricultural lands, organic and conventional agricultural practices) have created differences in microbial populations not only in the terms of variation of soil bacterial and fungal number, abundance and diversity but also regarding the represented fungal ecological groups and species composition of soil fungi.
4. In addition, number, abundance and diversity of soil microorganisms are significantly affected by the concentration of magnesium, potassium, phosphorus, nitrogen in the soil, C/N ratio and humic acid concentration.

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