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Molecular biology of soil fungi participating in litter decomposition in forest ecosystems

Molekulární biologie půdních hub, podílejících se na rozkladu opadu v lesních ekosystémech

Ph.D. Thesis

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Declaration

I declare that all sources and literature are properly cited and that the content of this thesis or its major part was not previously used for obtaining of the same or other academic degree.

Prague, 05.08.2013 Jana Voříšková

Table of contents

ACKNOWLEDGEMENTS 7

ABSTRACT 8

ABSTRAKT 10

LIST OF ABBREVIATIONS 13

LIST OF PUBLICATIONS 15

1 INTRODUCTION 17

1.1 TEMPERATE FOREST ECOSYSTEMS 17
1.2 PLANT LITTER COMPOSITION 18
1.3 PLANT LITTER DECOMPOSITION 19
1.4 DECOMPOSITION OF PLANT BIOPOLYMERS BY FUNGI 19
1.5 FUNGI IN FOREST SOIL 21
1.6 TEMPORAL AND SPATIAL SUCCESSION OF FUNGI IN FOREST SOIL 24
1.7 INFLUENCE OF LITTER COMPOSITION ON ITS DEGRADATION 26

2 AIMS 29

3 MATERIAL AND METHODS 31

4 DISCUSSION 33

5 CONCLUSIONS 43

6 REFERENCES 45

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Abstract

In forest ecosystems, substantial part of carbon enters soil in the form of plant litter. The decomposition of litter and soil organic matter represents an important process affecting nutrient cycling and carbon balance in soils. Fungi are considered the primary decomposers in terrestrial ecosystems due to the production of wide range of extracellular enzymes that allow them to attack the lignocellulose matrix in litter. Even if fungi represent key players in organic matter decomposition, the information about the structure and diversity of their communities is still limited and the roles of individual fungal taxa in forest soils remain unclear.

This Ph.D. thesis focused on the characterization of fungal communities in forest soils and their potential to decompose plant litter. The method for in-depth analysis of complex microbial communities from environmental samples was established and used. In addition, single eukaryotic functional gene was analysed in soil for the first time at a depth that allowed reliable estimation of diversity.

It was demonstrated that microbial community composition differs among horizons of forest soil profile. Despite similar diversity, significant differences in microbial community composition were observed between the DNA and RNA. Several microbial groups highly abundant in RNA pool showed only low abundance in DNA community indicating that low-abundance species make an important contribution to decomposition processes in soils. During plant litter decomposition, fungal community undergoes rapid succession with dramatic changes in its composition and most of the abundant taxa only temporarily dominate in the substrate. In forest soil, fungal activity, biomass and diversity decrease substantially with depth. The structure of fungal community in forest soil is distinctively influenced by the seasonal effects which are most apparent in the litter horizon. In the litter horizon, saprotrophic genera reached their seasonal maxima in autumn but summer typically saw the highest abundance of ectomycorrhizal taxa. While the composition of the litter community changed over the course of the year, the mineral soil rather showed changes in fungal biomass. Non-basidiomycetous fungi isolated from forest soil differed from saprotrophic basidiomycetes in their ability to decompose biopolymers present in litter and soil. Non-basidiomycetous fungi likely do not play significant role in lignin degradation but are able to produce a range of cellulolytic and chitinolytic enzymes giving the evidence that

they are actively engaged in decomposition of lignocellulose and dead fungal biomass. Concerning the effect of chemical composition of litter on its decomposition rate, it was demonstrated that litter nitrogen content positively correlates with litter mass loss while lignin content does not have any effect neither on the litter mass loss nor the activity of ligninolytic enzymes. This result suggests that the activity of ligninolytic enzymes is probably a less suitable indicator of lignin decomposition than expected.

Abstrakt

V lesních ekosystémech vstupuje významná část uhlíku do půdy ve formě rostlinného opadu. Dekompozice opadu a půdní organické hmoty je proto důležitým procesem ovlivňujícím bilanci živin a toky uhlíku v půdě. Houby jsou v terestrických ekosystémech pokládány za nejvýznamnější rozkladače a to díky své schopnosti produkovat řadu extracelulárních enzymů, které jim umožňují rozkládat biopolymery. I když houby zastávají klíčovou roli v procesu dekompozice, jen málo je známo o struktuře a diverzitě jejich společenstev a jejich přesná funkce v lesních půdách zůstává mnohdy nejasná.

Tato disertační práce byla zaměřena na charakterizaci houbových společenstev v lesních půdách a jejich schopností týkajících se dekompozice rostlinného opadu. Součástí této práce bylo vypracovat metodiku pro podrobnou analýzu komplexních mikrobiálních společenstev a využít ji pro analýzu environmentálních vzorků. Dále se podařilo kvantifikovat diverzitu genu pro exocelulázu v půdním vzorku.

Výsledky této práce ukázaly, že struktura mikrobiálního společenstva se liší mezi horizonty lesního půdního profilu. Významné rozdíly ve složení společenstva byly pozorovány mezi DNA a RNA komunitou navzdory jejich podobné diverzitě. Několik mikrobiálních taxonů vysoce abundantních v RNA vykazovalo jen velmi nízkou abundanci v DNA, což indikuje, že tyto druhy přes svoji nízkou početnost významně přispívají k dekompozičním procesům v půdách. Během dekompozice rostlinného opadu dochází k rychlým sukcesním změnám společenstva hub, přičemž většina abundantních druhů v substrátu dominuje pouze dočasně. Aktivita, množství biomasy a diverzita hub výrazně klesá s hloubkou půdy. Složení houbových společenstev v lesní půdě je výrazně ovlivněno sezónními vlivy, což je nejvíce patrné v nejsvrchnějším opadovém horizontu. V opadovém horizontu dosahují saprotrofní rody svého sezónního maxima na podzim, zatímco pro léto je typický nejvyšší výskyt ektomykorhizních hub. Minerální půdní horizont vykazuje významné sezónní změny v množství houbové biomasy. Houby izolované z lesní půdy se navzájem lišily schopností rozkládat půdní biopolymery. Houby nepatřící mezi saprotrofní basidiomycety pravděpodobně nehrají důležitou roli v rozkladu ligninu, ale jsou schopny produkovat řadu celulolytických a chitinolytických enzymů, což je předurčuje k aktivní roli při rozkladu lignocelulózy nebo mrtvé houbové biomasy. Při studiu vlivu chemického složení opadu na rychlost

jeho degradace, bylo ukázáno, že rychlost dekompozice stoupá s obsahem dusíku v opadu, zatímco obsah ligninu nemá vliv ani na úbytek hmotnosti, ani na aktivitu ligninolytických enzymů. Tento výsledek naznačuje, že aktivita ligninolytických enzymů je pravděpodobně méně vhodným indikátorem dekompozice ligninu než se předpokládalo.

List of Abbreviations

AM	arbuscular mycorrhiza
C	carbon
DNA	deoxyribonucleic acid
cDNA	complementary DNA
ECM	ectomycorrhiza
ITS	internal transcribed spacer
N	nitrogen
OTU	operational taxonomic unit
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA

List of Publications

This thesis consists of the following papers:

Paper I

Baldrian, P., Kolařík, M., Štursová, M., Kopecký, J., Valášková, V., Větrovský, T., Žifčáková, L., Šnajdr, J., Rídl, J., Vlček, Č., Voříšková, J. (2012) Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. ISME Journal 6: 248-258. IF_{2012} 8.93

Paper II

Voříšková, J., Baldrian, P. (2013) Fungal community on decomposing leaf litter undergoes rapid successional changes. ISME Journal 7: 477-486. IF_{2012} 8.93

Paper III

Voříšková, J., Brabcová, V., Cajthaml, T., Baldrian, P. Seasonal dynamics of fungal communities in a temperate oak forest soil. New Phytologist, *in press*. IF_{2012} 6.74

Paper IV

Baldrian, P., Voříšková, J., Dobiášová, P., Merhautová, V., Lisá, L., Valášková, V. (2011) Production of extracellular enzymes and degradation of biopolymers by saprotrophic microfungi from the upper layers of forest soil. Plant and Soil 338: 111-125. IF_{2012} 2.64

Paper V

Voříšková, J., Dobiášová, P., Šnajdr, J., Vaněk, D., Cajthaml, T., Šantrůčková, H., Baldrian, P. (2011) Chemical composition of litter affects the growth and enzyme production by the saprotrophic basidiomycete *Hypholoma fasciculare*. Fungal Ecology 4: 417-426. IF_{2012} 2.85

1 Introduction

1.1 Temperate forest ecosystems

Temperate forests belong among the major biomes on Earth, covering the area of 570 million ha (FAO and JRC, 2012). In forest ecosystems, important part of carbon enters the soil in form of plant litter (Berg and McClaugherty, 2003) which accumulates on the forest ground and is composed of leaves, needles, stalks, seeds and related organs, twigs and bark. For example in temperate deciduous forests, the mean annual plant litter input is estimated to be 3.5 $t.ha^{-1}$ (Bray and Gorham, 1964). Moreover, considerable proportion of the organic material is incorporated into soil as belowground input in the form of dead roots or by rhizodeposition (Kögel-Knabner, 2002).

Deciduous forests represent native vegetation in temperate zone and their trees are characterized by seasonally limited abscission of senescent leaves that is limited into autumn, when fresh litter with easily available nutrients accumulates on the forest floor (Šnajdr et al., 2011). On the other hand, coniferous forests are widely distributed in higher altitudes of temperate zone or represent a plantation forests in the same zone. In contrast to deciduous forests, coniferous trees tend to be evergreen, thus they shed needles throughout the year. The seasonal occurrence and changing intensity of photosynthetic activity of trees have been found to result in seasonality of belowground carbon flow and carbon availability to microbes in forest soil (Högberg et al., 2010; Kaiser et al., 2010). The rate of photosynthesis increases during the vegetative season and trees thus allocate higher amounts of assimilated carbon to underground in the autumn (Högberg et al., 2010).

In forest soils, as a consequence of new litter input and microbial activity, it is possible to recognize three main compartments of soil profile: litter horizon, containing almost exclusively organic matter derived from dead plant biomass (L), organic (humic) horizon, representing a mixture or processed plant-derived organic matter and soil components (H) and mineral soil horizon with low content of organic matter originating both from the organic matter decomposition and root exudation (Ah). The vertical position of organic material within the soil profile changes as decomposition progresses and the material moves downward. This is accompanied by the changes in its chemical

composition and particularly by the decrease of the content of utilizable carbon compounds (Šnajdr et al., 2008).

1.2 Plant litter composition

Plant litter consists of several major classes of organic compound. The relative amounts of the compounds vary among plant species as well as plant parts. In general, quantitatively the most common components in plant litter are polymer carbohydrates such as cellulose and hemicelluloses and the complex aromatic polymer lignin. Cellulose is the most abundant biopolymer on Earth (Schurz, 1999) and in plant litter it may constitute 10 - 50% of the litter mass. It is a polysaccharide consisting of a linear chain of several hundreds to over twenty thousands of $\beta(1\rightarrow4)$ linked D-glucose units. In contrast to cellulose, hemicelluloses are often heteropolymers derived from several monosaccharides most often glucose, xylose, mannose, galactose and arabinose. Hemicelluloses consist of shorter chains (between 70 - 200 sugar units) which are linear or branched. They make up as much as 20-40% of the plant litter (Berg and McClaugherty, 2003). Lignins are very heterogeneous and complex polymers, their molecules are linked by several different linkages and most of them are not readily hydrolysable. Lignins form heavily branched aromatic macromolecules containing phenolic constituents (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol). After cellulose, lignin is the second most abundant biopolymer (Boerjan et al., 2003) and comprises 15-40% of litter mass (Berg and McClaugherty, 2003). The molecule of lignin is water-insoluble, non-hydrolysable and highly resistant to microbial degradation in comparison with polysaccharides and other biopolymers. Lignin is closely associated with cellulose and hemicelluloses in plant cell walls and all of these compounds together form a complex referred to as lignocellulose (Evans and Hedger, 2001). Furthermore, variable proportion of plant litter is comprised of other compounds including pectins, tannins, cutin and proteins. These have various functions including protection of living leaves. Fresh litter also contains low-molecular-weight substances, such as amino acids, simple sugars and short-chain fatty acids.

1.3 Plant litter decomposition

Plant litter decomposition is the primary route through which nutrients return to the soil (Berg et al., 2001). It is a process involving mineralization and transformation including humus formation. Because most of the plant biomass-derived carbon in forests is mineralised in the litter and upper part of the soil, an understanding of this process and the microorganisms involved is the key for the identification of factors that affect global carbon fluxes. Furthermore, recycling of carbon and nutrients during the decomposition has an impact on the nutrient availability and consequently plant growth and community structure. Plant litter is a main source of energy and matter for a diverse community of soil microorganisms.

Litter decomposition in natural ecosystems is mainly driven by fungi, bacteria, and invertebrates (Hattenschwiler et al., 2005). In temperate forests fungi play a pivotal role in this process and especially saprotrophic basidiomycetes are considered to be the most important group of microorganisms involved in the breakdown and chemical conversion of litter components (Baldrian, 2008).

1.4 Decomposition of plant biopolymers by fungi

Fungi are able to produce large sets of extracellular degradative enzymes allowing them to attack the recalcitrant lignocellulose matrix. Cellulose and hemicellulose can serve as a sole source of energy and carbon. Lignin is a poor source of energy and polysaccharides serve as a co-substrate for its decomposition (Kirk et al., 1976). The decomposition of lignin opens the access to cellulose for microorganisms, exposes it to degradation and also leads to faster colonization of lignocellulotic substrate.

Fungal system of hydrolytic enzymes for efficient cellulose degradation usually consists of three enzymes: endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and 1,4-β-glucosidase (EC 3.2.1.21) (Baldrian and Valášková, 2008). Endoglucanases belong to endo-cleaving enzymes attacking long chains of cellulose and also shorter oligosaccharides. Exo-type enzymes cellobiohydrolases cleave cellulose fibers and release the disaccharide cellobiose which can be further hydrolysed by β-glucosidases into two glucose units. The resulting cellobiose and glucose molecules can be absorbed by fungal mycelium. Hemicellulose degradation is similar to cellulose

hydrolysis but it requires a larger set of different enzymes, because of the complex structure of hemicellulose. Endo-cleaving enzymes cleave long hemicellulose chains and release shorter fragments which are further degraded by exo-cleaving enzymes into small soluble compounds (Baldrian, 2008).

Lignin is highly resistant to the microbial decomposition and only a limited group of fungi is able to perform its complete decomposition however range of fungal strains can induce structural changes in lignins macromolecules (Hatakka, 1994). The fungal enzymatic system for lignin degradation in based on oxidative enzymes with wide substrate specificity. Ligninolytic set of enzymes is composed of oxidases, peroxidases and enzymes producing hydrogen peroxide. Laccases (EC 1.10.3.2) are copper-containing oxidases catalyzing oxidation of phenolic compounds. These enzymes are found in many fungal taxa (Baldrian, 2006). Class II peroxidases are secreted by several groups of basidiomycetous fungi (Hatakka, 1994) and include enzymes lignin peroxidases (EC 1.11.1.14), Mn-peroxidases (EC 1.11.1.13) and versatile peroxidases (EC 1.11.1.16) that catalyze oxidation of wide variety of aromatic macromolecules (including lignin and its related compounds). In addition, accessory enzymes such as glyoxalate oxidase (EC 1.2.3.5), glucose-1-oxidase (EC 1.1.3.4) and aryl alcohol oxidase (EC 1.1.3.7) generating hydrogen peroxide required by peroxidases have been found to be involved in lignin degradation (Martinez et al., 2005). Lignocellulose can be also degraded by nonenzymatic system - Fenton reaction, where hydroxyl radicals are produce and attack lignocellulose molecules, that leads into cellulose and hemicellulose degradation and modification of lignin molecule. The nonenzymatic system is important for group of wood-associated fungi that do not produce ligninolytic enzymes (Arantes et al., 2011).

Extracellular enzyme production in environmental samples is often analysed by enzyme assays that indicate the activity of microbial community. It is, however, not possible to directly link these observations to the activity of individual microbial taxa. Analysis of genes and transcripts encoding for degradative enzymes provides a tool for assessment of the role of individual taxa during the decomposition process. Moreover, it allows the determination of expression level, assessment of gene or transcript diversity and characterization of their spatial and temporal distribution. Several recent studies showed that genes and transcripts encoding for laccase, Mn-peroxidase, class II peroxidases, cellulolytic and other hydrolytic and oxidative enzymes can be analysed in environmental samples (Bodeker et al., 2009; Damon et al., 2012; Edwards et al., 2008;

Kellner and Vandenbol, 2010; Luis et al., 2005a; Luis et al., 2004; Uroz et al., 2013). For example genes and transcripts encoding for laccase have been widely studied by Luis et al. (2005a) who demonstrated that less than 30% of laccases genes is expressed in forest soil. Edwards et al. (2008) examined the diversity and distribution of the genes encoding for cellobiohydrolase (*cbhI*), the rate-limiting enzyme for the decomposition of cellulose, in forest soils.

1.5 Fungi in forest soils

Fungi are very diverse group of organisms playing a key role in many of the major processes in terrestrial ecosystems including organic matter decomposition, nutrient recycling and regulation of biogeochemical cycles that have subsequently an impact on bacterial, plant and animal communities. Fungi exhibit variety of growth forms and trophic strategies including saprotrophs obtaining organic compounds from dead organic matter, parasites (pathogens) attacking living tissues and symbionts with different strategies. Despite the fundamental role of fungi in many ecosystem processes, most of the available information concerning soil fungal communities is based on culture dependent approaches which are considered to be unrepresentative and selective because only a small fraction of total microbial population occurring in environmental samples is cultivable (Amann et al., 1995). The method of direct extraction of nucleic acids from environmental samples followed by PCR-based amplification of fungal ribosomal DNA, cloning of PCR fragments and subsequent sequence analysis has been used for characterization of fungal communities is forest soil ecosystems (Lindahl et al., 2007; O'Brien et al., 2005; Rosling et al., 2003). However, these methods underestimate community richness in complex populations and may omit rare but important species. Recently high-throughput sequencing technologies have opened up new dimension for study of soil fungal ecology. These methods have become widespread and are increasingly applied in environmental studies for assessment of fungal community composition e.g.: Buée et al. (2009), Hartmann et al. (2012) and Jumpponen et al. (2010). In addition, the knowledge of microbial communities in environment was until recently largely derived from the studies of DNA and no information is available on the relationships between the structure and diversity of this total community and community of active microbes assessed, for example, by targeting RNA molecules

(Anderson et al., 2008; Urich et al., 2008). The comparison of the DNA and RNA communities can also help to answer the question how well are the metabolically active microbial taxa represented in the common studies using the sequencing of soil DNA.

Saprotrophic fungi in forest soils are primarily responsible for degradation of organic matter. They are better adapted for plant litter decomposition than bacteria due to their metabolism, production of extracellular lignocellulolytic enzymes and capability to readily colonize new substrate as a result of their filamentous (hyphal) growth. A small group of saprotrophic basidiomycetes represents the exclusive organisms with the ability to completely degrade lignin molecules. On the other hand, the degradation of plant polysaccharides such as cellulose and hemicelluloses can be performed by many fungi belonging to multiple phyla (Chávez et al., 2006; Lynd et al., 2002; Štursová et al., 2012). However, the information about the involvement of nonbasidiomycetous fungi in organic matter decomposition remains still limited, even if these fungi represent highly abundant group in forest soils (O'Brien et al., 2005) and prevail during initial stages of litter decomposition (Osono, 2007).

In contrast to saprotrophs, mycorrhizal fungi are obligate symbionts obtaining carbon compounds derived from the photosynthates due to the association with the roots or other underground organs of autotrophic host plants (Hobbie, 2006). On the other hand, plants benefit from this association by obtaining soil-derived nutrients (mainly phosphorus and nitrogen) which are absorbed by fungi from the soil (van der Heijden and Horton, 2009). As a result, plants that are colonized by mycorrhizal fungi often grow much faster (Hoeksema et al., 2010), have a higher biodiversity (van der Heijden et al., 1998) or higher resistance to pathogens (Azcón-Aguilar and Barea, 1997). Clemmensen et al. (2013) have recently reported that mycorrhizal fungi contribute directly to the carbon enrichment of soils by mediating the belowground allocation of carbon from plant roots to soil. We can recognize several various types of mycorrhiza that differ in anatomy and physiology. Endomycorrhizas are characterized by penetration of fungus into the root cells and include arbuscular, orchid and ericoid mycorrhizas. Unlike other mycorrhizal relationships, ectomycorrhizal fungi typically do not penetrate into the plant host cells.

Arbuscular mycorrhiza (AM) is the most ancient and the most common form of mycorrhiza. Fungi involved in arbuscular mycorrhiza have been placed into a recently established monophyletic phylum, the *Glomeromycota* (Schüler et al., 2001). They are completely dependent on the sources of carbon from plants and their host plant range is

very wide. It is expected that AM could be found in 95% of vascular plant species in existence today (Gryndler et al., 2004) however they are most frequently associated with herbaceous plants and certain tropical trees. *Glomeromycota* form typical branched structures called arbuscules that penetrate into the plant root cells.

Ectomycorrhiza (ECM) is mainly formed between fungi belonging to *Basidiomycota* or *Ascomycota* and forest trees especially in temperate and boreal ecosystems. This type of mycorrhiza is characterized by a presence of a thick sheath of fungal tissue developed around terminal branches of plant roots. Fungal hyphae invade the plant roots and form there an intercellular network between the root cortical cells.

Even if mycorrhizal fungi are conventionally regarded as symbionts, their abilities to degrade organic matter and thus act as saprotrophs have been widely discussed (Baldrian, 2009; Cullings and Courty, 2009; Talbot et al., 2013). Several studies have reported genes encoding for degradative enzymes in genomes of mycorrhizal fungi (Bodeker et al., 2009; Luis et al., 2005b; Martin et al., 2008). Furthermore, Hibbett et al.(2000) showed that range of switches have occurred between symbiotic lifestyle and saprotrophy during evolution and both strategies can be represented in closely related fungal taxa, thus these two fungal groups can be more functionally similar than previously expected. For example Courty et al. (2006) and Vaario et al. (2012) have reported that mycorrhizal fungi are able to degrade some litter components due to the production of extracellular enzymes however enzymatic activities of the tested ectomycorrhizal strains were lower in comparison with saprotrophic fungi (Colpaert and vanTichelen, 1996). Although mycorrhizal fungi do not probably represent fundamental decomposers, it has been suggested that they might have an essential role in organic matter decomposition in forest ecosystems (Talbot et al., 2013). Mycorrhizal fungi in forest soil may affect litter decomposition due to the competition with saprotrophs for carbon and nutrients, especially for nitrogen. Nitrogen insufficiency in litter restricts the activity of litter decomposing fungi, thus the presence of mycorrhizal fungi may negatively influence decomposition rate (Gadgil and Gadgil, 1971).

1.6 Temporal and spatial succession of fungi in forest soil

Soil represents a complex environment inhabited by a wide range of microorganisms whose diversity is supposed to be extraordinarily high (Prosser, 2002). The structure and activity of fungal communities in forest soils is dependent on the occurrence of nutrients and varies during the process of degradation as the quality of the substrate changes (Osono, 2007). In forest soils the availability of nutrients distinctively differs through the soil profile (Šnajdr et al., 2008) thus the vertical stratification is characteristic feature of forest soils. The vertical position of litter-derived organic material in topsoil layer changes and its age increases with soil depth - old litter of uppermost horizon is replaced by that more recently fallen. This process leads to increasing recalcitrance of organic matter and the formation of humic compounds in the deeper horizons. The vertical distribution of fungal community in boreal and temperate forests has been well described by Lindahl et al. (2007) and O'Brien et al. (2005) who showed distinct spatial separation of saprotrophic and mycorrhizal communities. Saprotrophs were primarily confined to the surface of forest floor where carbon is mineralized, while mycorrhizal fungi dominated in the underlying more decomposed litter and humus where they mobilize nitrogen and make it available to plants (Lindahl et al., 2007; O'Brien et al., 2005).

Decomposition of leaf litter is a sequential process that initially involves the loss of the less recalcitrant components (for example, oligosaccharides, organic acids, hemicellulose and cellulose) followed by the degradation of the remaining highly recalcitrant compounds such as lignin. Litter quality changes during the course of its transformation and so does the activity of litter-associated microorganisms (Dilly et al., 2001) reflecting the varied catabolic capabilities that are sequentially required to complete the process of litter decomposition (Frankland, 1998; Osono, 2006). The knowledge about *in situ* fungal decomposition of plant litter is mainly based on isolation and identification of fungal decomposers (Koide et al., 2005b; Osono, 2005; Osono et al., 2009; Zhang et al., 2008). Using these cultivation-based approaches, fungi involved in the decomposition of litter have been divided into early, intermediate and late decomposers (Frankland, 1998; Tang et al., 2005). This observation has been recently supported by Šnajdr et al., (2011) who distinguished three phases of litter decomposition based on the differences in the activity of extracellular enzymes and the

rates of decomposition of the individual litter constituents. In previous culture-based studies fungi belonging to *Ascomycota* dominated during the initial stages of litter decay (Koide et al., 2005a) as they preferentially removed cellulose (Osono et al., 2003). Also a few basidiomycetes were found in the early stages of litter decomposition (Kubartova et al., 2009). The abundance of ascomycetous fungi decreased during the process of decomposition to be replaced by basidiomycetes in the later stages (Frankland, 1998; Osono, 2007). The degradation of plant leaves is not limited only to the litter layer on the forest floor. Indeed, the decomposition process already begins on attached plant leaves (Stone, 1987). Phyllosphere fungi that are established in the interior or on the surfaces of live leaves have the advantage of gaining access to readily available nutrients in live leaves and later, after senescence, to the dead leave biomass. There is some evidence that at least some of these fungi participate in litter decomposition (Korkama-Rajala et al., 2008; Žifčáková et al., 2011) but their importance in this process is still unclear.

Observations from diverse forest soils suggest that environmental factors such as temperature, water availability and substrate quality may be important factors affecting microbial community composition (Aponte et al., 2010; Kaiser et al., 2010; Kuffner et al., 2012; Landesman and Dighton, 2011). In deciduous forests, where the litterfall is limited to autumn, the irregular litter input is another factor influencing seasonal variations in microbial community composition. Moreover, seasonally fluctuating photosynthetic activity of trees leads to the seasonality of soil carbon allocation and its different availability to the soil biota in course of the year (Högberg et al., 2010; Kaiser et al., 2010). Prior studies concerning seasonal variations of fungal community were mainly based on traditional approaches such as enzyme assays or assessment of microbial biomass content (Baldrian et al., 2013; Criquet et al., 2002). There have thus far only been reports concerning specific functional groups of fungi (Courty et al., 2010; Courty et al., 2008) or comparing fungal communities on specific sampling occasions with limitations in particular soil or litter horizons (Coince et al., 2013; Davey et al., 2012; Dumbrell et al., 2011; Jumpponen et al., 2009). However, the knowledge of seasonal changes in overall fungal communities in temperate forest soil with respect to vertical stratification remains largely limited.

1.7 Influence of litter composition on its degradation

The chemical composition of litter varies among plant species and is known to affect rates of its decomposition (Hattenschwiler and Gasser, 2005). The differences in the decomposition rate are attributed to the litter quality such as leave tensile strength, lignin, carbon, nitrogen content, the carbon/nitrogen and lignin/nitrogen ratios and their impact on activity of microbes (Osono and Takeda, 2006; Pérez-Harguindeguy et al., 2000). The carbon: nitrogen ratio and concentration of lignin are regarded as the most important indicators of litter degradability by microorganisms (Liu et al., 2010; Zhang et al., 2008) because high carbon: nitrogen ratio and lignin content may prevent or slow down the microbial colonization of substrate. For instance beech (*Fagus sylvatica*), oak (*Quercus petraea*) and maple (*Acer campestre*) leaves with carbon:nitrogen ratios 60, 52 and 47 and lignin contents 10.2%, 10.0% and 10.9% (Hattenschwiler and Gasser, 2005) are considered to be slowly decomposing species in comparison with hornbeam (*Carpinus betulus*), wild cherry (*Prunus avium*) and lime (*Tilia platyphyllos*) leaves with carbon:nitrogen ratios 37, 45 and 30 and lignin contents 8.0%, 8.2% and 8.9% (Hattenschwiler and Gasser, 2005) which are species with faster decomposition rates. Dead plant organic matter is characterised by the relative excess of carbon and relatively low nitrogen and phosphorus contents. During the process of decomposition, carbon from freshly fallen litter is released as CO_2 and if the N is retained its relative proportion increases. For example, fresh *Quercus petraea* litter has a C/N ratio of 25, compared with 13-17 after *in vitro* degradation by saprotrophic fungi (Steffen et al., 2007). Under natural conditions these changes in litter quality are accompanied by changes in microbial community composition (see above). The development of microbial community composition has general pattern of fungal functional group (Osono, 2007; Šnajdr et al., 2011). Nevertheless, the successional series of dominant fungal species can be litter-type specific (Frankland, 1998; Koide et al., 2005b; Osono and Takeda, 2006) thus it is not simple to find the influence of litter composition on its degradation. In order to assess the relation between litter chemistry and decomposition without the effect of successive changes in microbial community composition, several studies followed the litter decomposition by single fungal species under defined laboratory conditions (Osono and Takeda, 2002, 2006; Steffen et al., 2007). However,

broader information about relationship between the roles of individual litter components on the decomposition rate remains still largely limited.

2 Aims

My Ph.D. thesis focuses on characterization of fungal communities in forest soils and their abilities to decompose plant litter. In order to get in-depth characterization of microbial communities in forest soils, it was aimed to establish the method of targeted amplicon library preparation for 454 pyrosequencing. Using this approach the biodiversity and structure of DNA and RNA-derived community of fungi and bacteria in spruce forest soil were analyzed (Paper I). Furthermore, it was demonstrated how the DNA and RNA communities differ and what part of the total community is metabolically active at a given moment. Because the soil sampling was performed under freshly fallen snow, in the period when decomposition processes in soil prevail, it was expected that decomposer microorganisms will be transcriptionally more active. It was also aimed to specifically target the important rate-limiting step in the process of cellulose decomposition by analyzing the genes and transcripts of the *cbhI* cellobiohydrolase (exocellulase). The experiment described in Paper II was aimed to provide a detailed characterization of the process of litter decomposition in forest soil. For this purpose the changes in fungal community composition and diversity were studied during 24 months of oak litter *in situ* decomposition. To specifically address the decomposition of cellulose, the composition of the gene pool of the *cbhI* was monitored. In order to evaluate the role of phyllosphere fungi in litter decomposition, fungal communities associated with live oak leaves and senescent leaves before their abscission were also analysed. It was expected that the structure of fungal community would reflect the availability of nutrients and the fungal diversity would increase during the process of decomposition as a consequence of the increase of substrate heterogeneity and the formation of new niches containing the dead biomass of early decomposers. Paper III studied the seasonal changes of fungal community in a forest soil. Considering the sharp and distinct vertical stratification, it was hypothesised that the structure of the fungal community would reflect the availability of nutrients in the horizons of soil profile. Based on the results obtained in Paper II, where considerable temporal shifts in fungal community structure during the decomposition of oak litter were observed, similar changes in the litter horizon were anticipated because the last year's litter represents a considerable percentage of the total litter mass. In the deeper horizons, a shift from a high relative abundance of ectomycorrhizal taxa during the

vegetative season to a high proportion of saprotrophic taxa in the absence of root C allocation was expected because Paper I showed that saprotrophic taxa were more metabolically active in the absence of photosynthesis carbon allocation belowground. In Paper IV it was aimed to compare the decomposition abilities of basidiomycetous and non-basidiomycetous fungi. Non-basidiomycetous fungi were isolated from the same study site that was studied in Paper II and Paper III because the direct analysis of DNA from this ecosystem showed that nonbasidiomycetous fungi represented considerable proportion of the entire fungal community. Paper V focused on the relationships between chemical composition of plant litter and its decomposition under laboratory conditions. To assess the relationships between litter chemistry and decomposition without the effects of temporary changes in microbial community composition, single fungal species (*Hypholoma fasciculare*) was grown on different types of litter that differed in chemical composition. It was hypothesized that the content of lignin in litter would cause high production of ligninolytic enzymes to increase the availability of carbohydrates while a high N content would inhibit lignin decomposition.

3 Materials and Methods

List of methods:

Soil sample collection

Cultivation of fungi

Enzyme assays

Quantification of fungal biomass

Taxonomic identification of fungal strains

Library preparation for tag-encoded amplicon pyrosequencing

Bioinformatic analysis of pyrosequencing data

Diversity and statistical analysis

4 Discussion

In order to get deeper insight into the fungal and bacterial community composition in forest soil the method of targeted amplicon library preparation for 454 pyrosequencing platform was established. This method comprises two-step PCR which is important for successful library preparation from complex DNA (cDNA) templates, such as nucleic acids extracted from soil samples (Paper I). Using this cultivation-independent deep sequencing approach, the biodiversity and structure of DNA as well as RNA-derived community of fungi and bacteria along with the diversity estimation of a functional eukaryotic gene involved in decomposition in spruce (*Picea abies*) forest soil was analyzed for the first time. Analysis of DNA-derived 16S rRNA and 18S rRNA gene sequences represents all bacteria and fungi present in the sample. RNA-derived sequences of bacteria represent the active part of the community that produce and contain ribosomes. In contrast to bacterial rRNA, analysis of fungal rRNA-ITS region offers a unique opportunity to target the precursor rRNA molecules. ITS regions are removed during the rRNA processing so this approach allow us to identify the species synthesizing ribosomes at a given moment and those which are likely metabolically active (Anderson et al., 2008). In order to specifically target an important decomposition process the gene and transcript pools of the gene *cbhI* (Edwards et al., 2008), encoding for cellobiohydrolase, which is an enzyme that catalyses the rate-limiting step in the decomposition of cellulose, were also analyzed and assigned to their producers. In Paper I it was demonstrated that diversity of bacterial community is considerably higher than the diversity of fungal population. When 1000 randomly selected sequences were analysed, 302-366 OTUs were found in bacteria, compared with 141-236 in fungi. Abundances of the major bacterial and fungal OTUs in different soils have been reported to be 2-3% and 7-17%, respectively (Buée et al., 2009; Fierer et al., 2007). In our study, where bacterial and fungal populations from the same soil were analyzed, bacterial communities were also more even than fungal ones and showed lower relative abundance of dominant species. While dominant bacterial species were distributed across the studied ecosystem, distribution of dominant fungi was often spatially restricted as they were only recovered at some locations. When comparing the uppermost litter horizon and underlying organic horizon of spruce forest soil, the litter horizon showed significantly higher fungal as well as bacterial biomasses in contrast to

deeper one (Figure 1, Paper I) that is in accordance with previous studies (Baldrian et al., 2013; Enowashu et al., 2009; Šnajdr et al., 2008). Microbial community composition differed among horizons of soil profile, thus bacteria and especially fungi together with *cbhI* clusters were often distinctly associated with a particular soil horizon. Bacterial sequences belonging into *Proteobacteria*, *Acidobacteria* and *Actinobacteria* were predominant in both horizons. In the litter horizon, the RNA community was enriched in *Acidobacteria* and *Firmicutes*, while in the organic horizon *Actinobacteria* were more abundant in the RNA community. The most abundant fungal orders in DNA-derived sequences were *Atheliales, Agaricales*, *Helotiales, Chaetothyriales* and *Russulales*. Members of *Botryosphaeriales*, *Lecanorales* and *Eurotiales* in the litter horizon and *Tremellales* and *Capnodiales* in the organic horizon were infrequent in the DNA communities but highly abundant among the RNA sequences (Figure 3, Paper I). Despite similar diversity of microbial communities based on DNA and RNA analysis, several microbial groups highly abundant in RNA pool, showed only low abundance in DNA pool suggesting that DNA-based surveys likely miss considerable portions of active microbial populations. Fungal community composition as well as *cbhI* clusters profoundly differed between the studied horizons of spruce forest soil in contrast to bacterial communities that showed less distinct vertical stratification. This observation thus highlight the importance of fungi in shaping the vertical structure of the forest soil. The among-horizon differences in fungal communities have been previously reported by Clemmensen et al. (2013), Lindahl et al. (2007), O'Brien et al. (2005) and Rosling et al. (2003). Substantial differences in fungal community assemblage among horizons of oak forest soil were also demonstrated in Paper III. In oak forest soil it was observed that the relative proportion of ectomycorrhizal taxa increased with soil depth however analysis of spruce forest soil showed that ectomycorrhizal fungi were highly abundant in both studied horizons, most likely due to the shallow rooting of *P. abies*. The most abundant genera of ectomycorrhizal fungi found in the spruce forest, *Piloderma* and *Tylospora* spp., are also the most abundant in the boreal *P. abies* forests in Finland and Sweden (Korkama-Rajala et al., 2008; Rosling et al., 2003; Wallander et al., 2010). In Paper I single eukaryotic functional gene at a depth that allowed diversity estimations was analysed for the first time. PCR amplicon sequencing represents the only feasible way to assess the diversity and distribution of functional genes because the proportion of these genes is very small in total DNA or RNA extracted directly from soil. For example, only nine

gene clusters of denitrification genes were obtained from 77 000 metagenome-derived clones (Demanèche et al., 2009). It was demonstrated that cellulose decomposition is mediated by highly diverse fungal populations which show distinct association with either the litter or organic horizons, indicating only a minor overlap of cellulolytic fungal communities between horizons. Litter horizon exhibited higher diversity and higher proportion of expressed sequences in contrast to humic horizon. Some of the most abundant *cbhI* sequences were transcribed by fungi with low abundances in the DNA-pool suggesting important contribution of low-abundance species to the decomposition processes in the soil. Distribution of amino-acid composition obtained by translation of *cbhI* sequences detected in spruce forest soil showed that internal peptide of *cbhI* varied in length and contained both conserved and highly variable regions (Figure 6, Paper I). Thus the deep amplicon sequencing of functional genes in environmental samples may improve the understanding of structure-function relationships and also contribute to the construction and evaluation of better primers and qPCR probes for targeting of these genes.

The decomposition of plant litter in forest floor is mainly mediated by fungal communities. Succession of fungi during litter decomposition was repeatedly studied by cultivation based methods e.g. Koide et al. (2005b), Osono et al. (2009) and Shirouzu et al. (2009). However, these successive changes have never been reported using cultivation independent deep sequencing approach. During 24 months of *Q. petraea* leaves decomposition, approximately 70 % of the total mass was lost (Figure 1, Paper II). Fungal biomass increased from low values in the live and senescent leaves to a maximum at month 2 and remained lower but constant until the end of the experiment. The activity of cellulolytic enzymes was detected in live and senescent leaves which indicate that decomposition started before leaf abscission (Figure 1, Paper II). Three distinct decomposition phases have been distinguished that are characterised by the sequential mass loss of extractables and hemicelluloses, cellulose, and lignin (Supplementary Figure 2, Paper II; Šnajdr et al. (2011)) In Paper II it was demonstrated that live oak leaves harboured a relatively rich fungal community with its diversity comparable to previous reports from *Quercus macrocarpa* (Jumpponen and Jones, 2009a). In senescent leaves the fungal community showed distinctive decrease of its diversity that was followed by rapid increase of number of fungal OTUs after the litterfall caused by the invasion of new colonizers. Fungal diversity continued to increase until month 4, indicating the arrival of new species on the substrate (Figure 1,

Paper II). Contrary to our expectations, the increase of substrate heterogeneity and the formation of new niches containing the dead biomass of early decomposers in later stages did not lead to the further increase of fungal diversity despite the dramatic changes in the community composition (Figure 2, Paper II). Live and senescent leaves on the trees were dominated by phyllosphere fungi belonging to *Ascomycota* (Figure 3, Paper II) which is in accordance with previous culture-based studies (Osono, 2002; Santamaría and Bayman, 2005) as well as with pyrosequencing analyses of live *Q. macrocarpa* leaves (Jumpponen and Jones, 2009a, b). In our study we showed that phyllosphere fungi were still quantitatively important during the subsequent stages of decomposition at least until month 4 (Figure 2 and 4, Paper II). The fact that some live leaf-associated fungi are able to produce extracellular enzymes or decompose sterile senescent leaves (Korkama-Rajala et al., 2008; Žifčáková et al., 2011) led to the hypothesis that certain taxa may change from comensalism to a saprotrophic strategy. The first year of our experiment in Paper II was characterised by a relatively rapid loss of litter mass, a decrease in the C/N ratio and the cellulose content, and a relatively high activity of cellulolytic enzymes which caused faster decomposition of cellulose (Supplementary Figure 2, Paper II). These conditions were associated with the continuous dominance of fungi from the *Ascomycota* phylum which are mostly able to degrade cellulose and hemicelluloses, while their ability to convert lignin is limited (Martinez et al., 2005). Dominance of ascomycetous fungi in the early stages of beech litter decomposition was recently also demonstrated using metaproteomic approach (Schneider et al., 2012). During the second year, the rate of litter mass loss was relatively slow and the activity of cellulolytic enzymes decreased which indicated that the easily accessible polysaccharides were depleted. Also, the substrate was richer in the recalcitrant lignin and nitrogen and characteristic with the increased activity of ligninolytic enzymes (Supplementary Figure 2, Paper II). Fungi from the *Basidiomycota* phylum distinctively prevailed over fungi from the *Ascomycota* phylum at month 24. In previous studies, basidiomycetous species have often been demonstrated to be dominant in the late stages of litter decomposition (Duong et al., 2008; Osono, 2007) because of their capability to synthesize enzymes required for the degradation of complex polymers remaining in decomposed litter (Baldrian, 2008). Relatively high diversity of *cbhI* genes was demonstrated in senescent leaves (58 observed and >200 predicted OTUs). Later in decomposition, estimates of *cbhI* richness were approximately 200 in number which indicate that there may be some 100 cellulolytic fungal species when multiple copies of

the gene per fungal genome are considered (Edwards et al., 2008; Weber et al., 2011). The overall fungal diversity did not correlate with the diversity of fungi harbouring the *cbhI* gene suggesting that the proportion of cellulolytic fungi changed during decomposition. In Paper II it was demonstrated that fungal succession during litter decomposition was much faster than so far expected from the culture-based studies (Frankland, 1998; Osono, 2007; Osono and Takeda, 2001; Tang et al., 2005) that divided fungi only into early, intermediate and late decomposers. The fast appearance–disappearance of fungal taxa seems to contrast with the reported persistence of DNA from inactive fungi in decaying wood (Rajala et al., 2011) and to support the rapid turnover of saprotrophs associated with various decomposition stages (Lindahl and Finlay, 2006). Distinct successive changes were also apparent in the community of cellulolytic fungi even if cellulose represents a substrate that is present in the litter during the entire decomposition process. Successional changes are thus likely governed not only by the relatively slow changes of the polysaccharides, lignin and nitrogen content in litter but possibly by other factors including more subtle changes in litter chemistry or interspecific fungal interactions.

Nutrients obtained due to the litter decomposition are not the sole energy source for fungal communities. Also photosynthates that are allocated belowground represent important resource (Högberg et al., 2008). Changes in photosynthetic activity of trees, the amount and quality of litter as well as other environmental factors differ across the seasons of the year and thus likely impact the structure of soil communities. Considering the sharp and distinct vertical stratification of temperate forest soil, it was hypothesised that the structure of the fungal community would reflect the availability of nutrients in the horizons of soil profile. Based on the study in Paper II where considerable temporal shifts in fungal community structure during the decomposition of oak litter were observed, similar changes in the litter horizon were anticipated because the last year's litter represents a considerable percentage of the total litter mass. In the deeper horizons, a shift from a high relative abundance of ectomycorrhizal taxa during the vegetative season to a high proportion of saprotrophic taxa in the absence of root C allocation was expected because in Paper I it was shown that saprotrophic taxa are more metabolically active during the period when photosynthesis does not occur. In Paper III it was demonstrated that fungal community in forest soil shows considerable vertical stratification; its activity, biomass and diversity substantially decreased with soil depth and its structure distinctively differed among the three horizons studied. As

demonstrated in Paper V, C/N ratio decreases during the process of decomposition. In previous study of Yang and Luo (2011) the decrease of C/N ratio with soil depth has been reported. However, in our study the highest C/N ratio was observed in the Ah horizon (the deepest studied horizon), a fact that might support the importance of the allocation of C from tree roots and thus considerable enrichment of deeper soil as proposed by Clemmensen et al., (2013) who demonstrated that 70% of soil carbon in boreal forest was root-derived and immobilized in the soil by mycorrhizal fungi. Interestingly, our study showed a substantial and significant decrease in community diversity: the amount of OTUs representing 80% of the fungal community was in average 90 in the litter horizon, 51 in the organic horizon and 25 in the Ah horizon (Figure 1, Paper III). It is in contrast with Paper I and study of O'Brien et al. (2005) where no significant changes in fungal diversity among horizons were observed. Comparing the relative abundance of major functional fungal groups among soil horizons, the litter horizon was predominantly colonized by saprotrophic fungi. The relative abundance of ectomycorrhizal basidiomycetes increased with soil depth (Figure 2, Paper III), even if their proportion in deciduous forest soil studied was lower than in spruce forest soil (Paper I). Our results concerning vertical distribution of fungal functional groups are in accordance with previous studies (Clemmensen et al., 2013; Lindahl et al., 2007; O'Brien et al., 2005). However, interestingly, if the absolute amount of fungal biomass is considered, the highest ECM biomass per g of soil dry mass, despite its lower proportion, was present in the litter horizon. Fungal community in deciduous forest soil also showed distinct seasonal variation. The majority of studied enzymes showed the highest activity in winter in all horizons but being most pronounced in the litter (Figure 1, Paper III). It is not surprising because temperate deciduous forests are characterised by seasonal input of large amount of fresh litter, which is limited to autumn, representing nutrient-rich substrate for microbial communities (Šnajdr et al., 2011). High enzyme activities in winter were followed by the increase of fungal biomass in the spring with a relative increase in the proportion of non-mycorrhizal taxa (Figure 1, Paper III). Fungal community composition in the litter horizon was distinctively more affected by the season than the deeper layers. In litter horizon saprotrophic fungal genera *Mycena*, *Mycosphaerella* and *Naevala* showed 30×, 200× and 350× differences in abundance among seasons. This result partly reflects succession on oak litter described in Paper II, for example *Mycosphaerella,* which peaked in autumn, is typical of senescent and freshly fallen oak leaves. Additionally, the

fungi that increased in winter, *Naevala*, *Rhodotorula* and *Cryptococcus*, were found to be associated with litter decomposition approximately 4 months after abscission (Paper II) and thus seem to be supported nutritionally by the last year's litter. The most abundant genera in litter layer represented saprotrophic *Mycena* which significantly peaked its abundance in spring when nutrient-rich substrate is available. Summer was characterised by dramatic increase of ectomycorrhizal fungi, for example *Amanita*, *Lactarius* and *Russula* increased their abundance 68x, 20x and 7x, respectively, if compared with spring. ECM fungi that were predominant in summer or autumn samples from litter horizon are known to produce large sporocarps which require high nutrients input. Högberg et al. (2010) recently reported that the production of sporocarps of ECM fungi is directly dependent on allocation of photosynthates in the late summer. Thus sporocarp formation and subsequent sporulation, which occur on the forest floor, can explain seasonal shifts of ECM community in litter horizon and relatively invariable ECM community in soil layer. Increasing abundance of ECM fungi in late summer or autumn in organic and soil horizons of boreal forest has been previously reported by Wallander et al. (2001) as well as by Davey et al., (2012) who studied bryophyte-associated fungal communities. In contrast to litter horizon, which showed profound seasonal changes in fungal community composition, Ah horizon rather responded in changes in fungal abundance however still approximately 30 % of the dominant fungal taxa exhibited seasonal patterns of occurrence. Contrary to our expectations, the relative proportion of saprotrophic fungi in Ah horizon did not increase during winter even though the increase of enzyme activities was detected in this season. One of the possible explanations might be the temporal switch of certain ECM taxa to a saprotrophic lifestyle allowing them to preserve their biomass. The fungal biomass content in the Ah horizons increased approximately threefold from spring to summer which corresponds with the expected increase in photosynthate allocation belowground. The unexpected observation that the proportion of ECM fungi does not increase during this season might indicate that root-supplied carbon can be used by both ECM and by saprotrophs in the soil. The findings in Paper III indicate that both litter decomposition and photosynthate allocation represent important factors contributing to the seasonal changes in fungal communities. Thus it was demonstrated that our understanding of the fungal community composition in ecosystems where environmental factors show seasonal variation is limited if this phenomenon is not considered.

Even if saprotrophic basidiomycetes are considered to be the most important litter decomposers (Baldrian, 2008), non-basidiomycetous fungi also likely play significant role in this process, if it is taken into account that they represent considerable proportion of litter-associated fungal community. Based on direct extraction of DNA from environmental samples and its subsequent analysis *Ascomycota* distinctively prevailed during the initial stages of litter decomposition (Paper II) and represented 42 % and 25 % of fungal community in litter and organic horizon, respectively, in oak forest soil (Paper III); the same study site that was used for isolation of fungal strains in Paper IV. More than half of the isolates belonged to the genus *Penicillium*, *Acremonium*, *Cladosporium*, *Geomyces*, *Mucor* and *Trichoderma*. These fungi have been repeatedly isolated from both forest and agricultural soils (De Bellis et al., 2007; Grishkan, 1996; Keller and Bidochka, 1998). However, contrary to our expectation, isolated fungal genera represented only a small fraction of ascomycetes detected in Paper III. In Paper IV only six non-basidiomycetous isolates from 29 tested showed phenol oxidation activity which was low and all of the non-basidiomycetous strains showed lack of ligninolytic peroxidases that sharply contrasted with the high activity of ligninolytic enzymes in all tested basidiomycete strains. For example, Claus and Filip (1998) and Zheng et al. (1999) have previously reported that ascomycetous *Cladosporium cladosporoides* and a *Penicillum* strain may be able to degrade lignin-like compounds however the identity of the enzymes involved remained unanswered. In Paper IV only limited production of hemicellulose-degrading enzymes by non-basidiomycetous fungi was reported that is in contrast with relatively high production of these enzymes by saprotrophic basidiomycetes (Steffen et al., 2007; Valaskova et al., 2007). On the other hand, non-basidiomycetous fungi were able to produce cellulolytic enzymes and the production of chitinases was in average higher than in basidiomycetes (Table 1 and Figure 1, Paper IV). Paper IV also demonstrated that the levels of production of cellulolytic enzymes differ greatly among strains of the same genus. Cellulose degradation has been previously demonstrated for isolates of ascomycetous *Penicillim* sp., *Acremonium* sp. and *Trichoderma* sp. (Hao et al., 2006). The occurrence of one or more cellulolytic enzymes was reported in individual members of ascomycetous genera *Acremonium*, *Cladosporium*, *Mucor*, *Myrothecium*, *Penicillium* and *Trichoderma* (Bhiri et al., 2008; Ikeda et al., 2007; Skare et al., 1975; Somkuti, 1974; Whitaker, 1951) and fungi belonging to the genera *Geomyces* and *Hypocrea* were found to be able to utilize cellulose (Štursová et al., 2012). Also chitinases have been

previously purified from ascomycetous *Mucor*, *Myrothecium*, *Penicillium* and *Trichoderma* (de la Cruz et al., 1992; Rast et al., 1991; Vyas and Deshpande, 1989). Considering the growth rates on individual substrates, the most important components of cellulose, hemicelluloses and chitin (cellobiose, xylose and N-acetylglucosaminidase) supported rapid growth of most of the non-basidiomycetous isolates (Figure 2, Paper IV).

Plant litter decomposition is performed by complex microbial communities that rapidly change during succession, as demonstrated in Paper II. However, the differences in litter composition lead to the differences in microbial community (Frankland, 1998; Osono and Takeda, 2006) thus it is difficult to find the relative role of individual chemical quality parameters on decomposition. The use of a single species in a decomposition test provides an experimental tool to assess the relationships between litter chemistry and decomposition without the effects of temporary changes in microbial community composition. It was hypothesized that the content of lignin in litter would cause high production of ligninolytic oxidases and peroxidases to increase the availability of carbohydrates while a high N content would inhibit lignin decomposition. In Paper V it was shown that during 12-week growth of the saprotrophic basidiomycete *Hypholoma fasciculare* on 11 types of litter with variable chemical composition, the litter mass loss ranged from 16% to 34% (Figure 1, Paper V). These values are comparable with 19-44% litter mass loss in birch litter after 3 months decomposition by *Mycena* and *Collybia* species (Osono and Takeda, 2006) or several litter-decomposing fungi on *Quercus petraea* litter (Steffen et al., 2007; Valaskova et al., 2007). During early decomposition stages increasing initial N content of the litter positively correlated with litter mass loss and ergosterol content (Figure 3, Figure 4, Paper V). However, contrary to our expectation lignin content did not affect litter mass loss and was not an important factor determining fungal growth. Positive corelation of N content with litter mass loss and ergosterol content is in contrast with earlier observations on synthetic media where both organic and mineral N inhibited the growth of certain saprotrophic basidiomycetes (Keyser et al., 1978) but in agreement with a previous study on *Mycena epipterygia* on pine litter where N addition increased substrate use efficiency and C addition had no effect (Boberg et al., 2008). In addition to litter mass loss, a significant part of the litter up to >30 % of the decomposed litter mass was transformed into fungal biomass in Paper V which is in agreement with *in situ* studies of Frankland (1998) and Frey et al. (2003). The initial loss of litter mass

significantly positively correlated with the activities of arylsulfatase, cellobiohydrolase, endoxylanase and phosphatase, the highest activity of ligninolytic enzymes were mostly detected at the beginning of the experiment (Supplementary Material, Paper V). Thus contrary to our expectation activities of most studied enzymes did not reflect the changes in litter quality as demonstrated in Paper II (Figure 1) and in the study of Šnajdr et al. (2011) where cellulolytic enzymes peaked at the beginning of litter decomposition while later stages of decomposition were characterized by high activities of ligninolytic enzymes. In Paper V it was shown that neither nitrogen nor lignin content has an impact on the activities of ligninolytic enzymes. The loss of lignin was not correlated with laccase or Mn-peroxidase activity even if these enzymes are considered to play crucial role in lignin decomposition (Baldrian, 2006; Hofrichter, 2002). Low lignin removal in litters with high Mn-peroxidase activity might have been caused by low activity of auxiliary enzymes that are responsible for H_2O_2 production whereas high lignin removal rates in litters with low activity of ligninolytic enzymes indicates that other mechanisms of lignin decomposition, possibly based on the production of reactive oxygen radicals (Baldrian and Valaskova, 2008), may be active in *Hypholoma fasciculare*. Thus in this study it was demonstrated that the activity of ligninolytic enzymes is probably a less suitable indicator of lignin decomposition then previously expected.

5 Conclusions

This work consists of 5 papers that contribute to the understanding of the composition of soil fungal communities and their role in the process of plant litter decomposition. The method for in-depth analysis of complex microbial communities from environmental samples was established and for the first time single eukaryotic functional gene at a depth that allowed diversity estimations was analysed in soil samples.

In a spruce forest it was demonstrated that microbial community composition differs among horizons of soil profile and certain bacteria and especially fungi together with *cbhI* gene clusters are often associated with a particular soil horizon. Despite similar diversity of microbial communities based on DNA and RNA analysis, significant differences in community composition were observed. Several microbial groups highly abundant in RNA pool showed only low abundance in DNA community indicating that low-abundance species make an important contribution to decomposition processes in soils.

Plant litter decomposition is highly complex process mediated by various fungal groups that undergo rapid succession with dramatic changes in the community composition. Furthermore, most of the abundant taxa only temporarily dominate in the substrate. Phyllosphere fungi comprise a significant proportion of the community during early decomposition.

Activity, biomass and diversity of fungal community decreases substantially with soil depth and its structure distinctively differs among the three horizons studied. The litter community exhibits profound seasonal changes. Abundance of the saprotrophic genera reaches their seasonal maximum in autumn, while summer typically saw the highest abundance of ectomycorrhizal taxa. While the composition of the litter community changes over the course of the year, the mineral soil shows changes in biomass. It seems that fungal community composition is mainly affected by the progress off litter decomposition together with phytosynthate allocation.

Soil non-basidiomycetous fungi differ from saprotrophic basidiomycetes in their ability do decompose soil biopolymers. Nonbasidiomycetous fungi do not likely play significant role in lignin degradation but are able to produce wide range of cellulolytic and chitinolytic enzymes giving the evidence that they are actively engaged in

decomposition of lignocellulose and dead fungal biomass. In a decomposition experiment including a single saprotrophic basidiomycete, decomposition rate depended on litter nitrogen content. Lignin content did not have any effect neither on the decomposition rate nor the activity of ligninolytic enzymes indicating that the activity of these enzymes is probably a less suitable indicator of lignin decomposition then expected.

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ORIGINAL ARTICLE

Active and total microbial communities in forest soil are largely different and highly stratified during decomposition

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Soils of coniferous forest ecosystems are important for the global carbon cycle, and the identification of active microbial decomposers is essential for understanding organic matter transformation in these ecosystems. By the independent analysis of DNA and RNA, whole communities of bacteria and fungi and its active members were compared in topsoil of a *Picea abies* forest during a period of organic matter decomposition. Fungi quantitatively dominate the microbial community in the litter horizon, while the organic horizon shows comparable amount of fungal and bacterial biomasses. Active microbial populations obtained by RNA analysis exhibit similar diversity as DNA-derived populations, but significantly differ in the composition of microbial taxa. Several highly active taxa, especially fungal ones, show low abundance or even absence in the DNA pool. Bacteria and especially fungi are often distinctly associated with a particular soil horizon. Fungal communities are less even than bacterial ones and show higher relative abundances of dominant species. While dominant bacterial species are distributed across the studied ecosystem, distribution of dominant fungi is often spatially restricted as they are only recovered at some locations. The sequences of *cbhI* gene encoding for cellobiohydrolase (exocellulase), an essential enzyme for cellulose decomposition, were compared in soil metagenome and metatranscriptome and assigned to their producers. Litter horizon exhibits higher diversity and higher proportion of expressed sequences than organic horizon. Cellulose decomposition is mediated by highly diverse fungal populations largely distinct between soil horizons. The results indicate that low-abundance species make an important contribution to decomposition processes in soils.
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Introduction

Most terrestrial ecosystem functions occur in the soil, which possesses the greatest amount of biodiversity on Earth. Yet, the understanding of how ecosystem functions are influenced by soil biodiversity is far behind our understanding of how aboveground organisms contribute to these functions (Bowker *et al.*, 2010). Soil microorganisms represent a considerable fraction of the living biomass on Earth, with 10^3–10^4 kg of microbial biomass per hectare of surface soils (Fierer *et al.*, 2007). In addition, microbial community composition is now recognised as an important determinant of ecosystem process rates (Reed and Martiny 2007; Strickland *et al.*, 2009). Understanding the structure and function of soil microbial communities is thus central to predicting how ecosystems will respond to future environmental conditions.

While several recent studies have used deep sequencing approaches to assess the diversity of soil bacterial components (Roesch *et al.*, 2007; Lauber *et al.*, 2009), the number of such studies addressing fungal diversity is still limited. This is true despite the fact that fungi comprise a large proportion of soil microbial biomass and have a dominant role in decomposition and nutrient cycling in soil (Bailey *et al.*, 2002; Buée *et al.*, 2009). Only a minor fraction of the estimated 1.5 million fungal species worldwide have been described (Hawksworth, 2001), and the ecological roles of most fungal taxa are poorly understood since the complexity of fungal communities has

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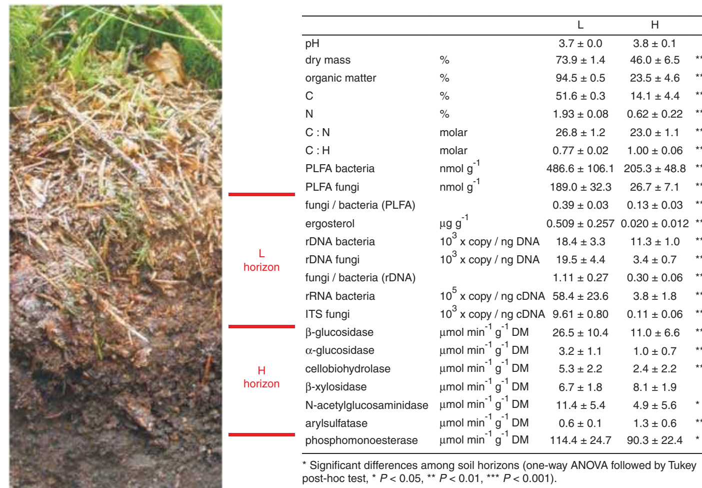

		L	H	
pH		3.7 ± 0.0	3.8 ± 0.1	
dry mass	%	73.9 ± 1.4	46.0 ± 6.5	***
organic matter	%	94.5 ± 0.5	23.5 ± 4.6	***
C	%	51.6 ± 0.3	14.1 ± 4.4	***
N	%	1.93 ± 0.08	0.62 ± 0.22	***
C : N	molar	26.8 ± 1.2	23.0 ± 1.1	***
C : H	molar	0.77 ± 0.02	1.00 ± 0.06	***
PLFA bacteria	nmol g^{-1}	486.6 ± 106.1	205.3 ± 48.8	**
PLFA fungi	nmol g^{-1}	189.0 ± 32.3	26.7 ± 7.1	***
fungi / bacteria (PLFA)		0.39 ± 0.03	0.13 ± 0.03	***
ergosterol	μg g^{-1}	0.509 ± 0.257	0.020 ± 0.012	***
rDNA bacteria	10^3 x copy / ng DNA	18.4 ± 3.3	11.3 ± 1.0	***
rDNA fungi	10^3 x copy / ng DNA	19.5 ± 4.4	3.4 ± 0.7	***
fungi / bacteria (rDNA)		1.11 ± 0.27	0.30 ± 0.06	***
rRNA bacteria	10^5 x copy / ng cDNA	58.4 ± 23.6	3.8 ± 1.8	***
ITS fungi	10^3 x copy / ng cDNA	9.61 ± 0.80	0.11 ± 0.06	***
β-glucosidase	μmol min^{-1} g^{-1} DM	26.5 ± 10.4	11.0 ± 6.6	***
α-glucosidase	μmol min^{-1} g^{-1} DM	3.2 ± 1.1	1.0 ± 0.7	***
cellobiohydrolase	μmol min^{-1} g^{-1} DM	5.3 ± 2.2	2.4 ± 2.2	**
β-xylosidase	μmol min^{-1} g^{-1} DM	6.7 ± 1.8	8.1 ± 1.9	
N-acetylglucosaminidase	μmol min^{-1} g^{-1} DM	11.4 ± 5.4	4.9 ± 5.6	*
arylsulfatase	μmol min^{-1} g^{-1} DM	0.6 ± 0.1	1.3 ± 0.6	**
phosphomonoesterase	μmol min^{-1} g^{-1} DM	114.4 ± 24.7	90.3 ± 22.4	*

* Significant differences among soil horizons (one-way ANOVA followed by Tukey post-hoc test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 1 Properties of *Picea abies* forest soil, abundance of microorganisms and activity of extracellular enzymes involved in organic matter decomposition in the L and H horizons. The data represent mean values and s.d. from four studied sites.

so far limited our ability to estimate diversity and distinguish individual taxa (McGuire and Treseder, 2010). In order to understand the soil ecosystem processes, it is essential to address the fungal and bacterial community at the same time. In addition, the ecology of total microbial communities is so far largely derived from the studies on DNA and no information is available on the relationships between the diversity of this total community and community of active microbes assessed, for example, by targeting the RNA molecules (Anderson and Parkin, 2007; Urich *et al.*, 2008).

From the global viewpoint, the understanding of fungal and bacterial diversity is highly important in the biomes of coniferous forests, where fungi quantitatively dominate bacteria in decomposing litter material, while the importance of bacteria increases with soil depth (Bååth and Anderson 2003). Coniferous forest ecosystems have a prominent role in the global carbon cycle (Myneni *et al.*, 2001), and knowledge of microbially mediated soil functions is thus required for estimating global C fluxes and their potential future changes. Forests dominated by spruce (*Picea* spp.) constitute large ecosystems in boreal forest biomes and are also widely distributed in higher altitude forests and plantation forests in the northern temperate zone.

It was recently proposed that the analyses of soil microbial community composition should be based on direct analysis of total RNA to avoid PCR bias (Urich *et al.*, 2008). While this approach may be feasible for bacterial community analyses, the sequence information contained in fungal rRNA molecules is insufficient for species discrimination; thus, internal transcribed spacer (ITS) regions of the rRNA are used instead. Because there are 10^3–10^4 times fewer fungal ITS sequences than bacterial 16S rRNA gene sequences in soil (Figure 1), amplification of fungal ITS is inevitable to achieve reasonable sampling depth. Here, we combined the analysis of DNA-derived bacterial 16S rRNA gene sequences representing all bacteria present and the RNA-derived sequences representing the content of bacterial ribosomes reflecting thus the active part of the total community. The analysis of fungal ITS1 and ITS2 sequences offers a unique opportunity to target the precursor rRNA molecules with fast turnover, thus identifying these species synthesising ribosomes at a given moment and thus likely metabolically active (Anderson and Parkin, 2007). The comparison of the DNA and RNA communities can also help to answer the question how well are the metabolically active microbial taxa represented in the common studies using the sequencing of soil DNA.

The aim of this work was to demonstrate how the DNA and RNA communities differ and what part of the total community is metabolically active at a given moment. The study was performed at the beginning of winter under freshly fallen snow to target the period when decomposition processes in soil prevail. In a mountainous *Picea abies* forest in central Europe where mycorrhizal fungi have a major role, the winter period without photosynthate flow is expected to show increased activity of decomposer fungal species. Litter and the organic horizons were studied separately because fungal and bacterial communities were previously found to differ between these horizons due to the differences in nutrient availability and the presence of root-associated microorganisms (O'Brien *et al.*, 2005; Lindahl *et al.*, 2007; Šnajdr *et al.*, 2008).

We expected that during the decomposition period, decomposer microorganisms will be transcriptionally

active and a large proportion of decomposition-related genes will be expressed by dominant taxa of microbial decomposers. In order to specifically target an important decomposition process, the gene and transcriptome pools of the fungal cellobiohydrolase (exocellulase) gene *cbhI* sequences were compared, as its gene product catalyses the rate-limiting step in the decomposition of cellulose, the most abundant biopolymer in the ecosystem (Baldrian and Valášková, 2008). The *cbhI* gene occurs in both *Ascomycota* and *Basidiomycota*, and it is also common in the genomes of saprotrophic fungi (Edwards *et al.*, 2008; Ohm *et al.*, 2010). While some recent studies showed that expression of eukaryotic decomposition-related genes in soils can be analysed (Luis *et al.*, 2005; Kellner and Vandenbol, 2010), only the DNA/RNA approach can answer the questions on the diversity of decomposer communities and the proportion of expressed genes. Since cellulose is present in both the litter and soil organic horizon (Šnajdr *et al.*, 2011), the same cellulose-decomposing microorganisms can be present and potentially active in both horizons. However, the higher amount of cellulose in litter likely supports higher diversity of cellulose decomposers.

Materials and methods

Study site, sample collection and soil analysis

Study area was located in the highest altitudes (1170–1200 m) of the Bohemian Forest mountain range (Central Europe) and was covered by an unmanaged spruce (*P. abies*) forest (49°02.64 N, 13°37.01 E). Sampling was performed in late October 2009 under freshly fallen snow (8–12 cm, 3 days after the snowfall) at −5 °C. At four sites, located 250 m from each other, six topsoil samples located around the circumference of a 4-m-diameter circle were collected. Litter horizon (L) and organic (humic) horizon (H) material were separately pooled. After removal of roots, L material was cut into 0.5 cm pieces and mixed; H material was passed through a 5-mm sterile mesh and mixed. Aliquots for nucleic acids extraction were immediately frozen and stored in liquid nitrogen. Samples for phospholipid fatty acid and ergosterol analysis were frozen and stored at −45 °C until analysis. Enzyme assays were performed within 48 h in samples kept at 4 °C in soil homogenates (Štursová and Baldrian, 2011). Dry mass content was measured after drying at 85 °C, organic matter content after burning at 650 °C and pH was measured in distilled water (1:10). Soil C and N content was measured using an elemental analyser.

Quantification of microbial biomass

Phospholipid fatty acid was extracted by chloroform–methanol–phosphate buffer, subjected to alkaline methanolysis and free methyl esters were analysed by GC-MS (Šnajdr *et al.*, 2008). Fungal biomass was quantified based on 18:2ω6,9 content, and bacterial biomass as the sum of bacteria-specific phospholipid fatty acid (Bååth and Anderson, 2003). Total ergosterol was extracted with 10% KOH in methanol and analysed by HPLC (Šnajdr *et al.*, 2008). Partial bacterial and fungal rDNAs were quantified by qPCR using 1108f and 1132r primers for bacteria (Wilmotte *et al.*, 1993; Amann *et al.*, 1995) and ITS1/qITS2* primers for fungi (White *et al.*, 1990; Šnajdr *et al.*, 2011).

Nucleic acid extraction and reverse transcription

RNA and DNA were co-extracted using the RNA PowerSoil Total RNA Isolation Kit and DNA Elution Accessory Kit (MoBio Laboratories, Carlsbad, CA, USA) combined with the OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA). Three soil aliquots (3 × 3 g of material) were extracted per sample. Extracted RNA was treated with DNase I and 1 μg was reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primers. Samples were designated as LD = litter DNA, LR = litter cDNA, HD = humic horizon DNA and HR = humic horizon cDNA.

Tag-encoded amplicon pyrosequencing and sequence analysis

The eubacterial primers eub530F/eub1100aR (modified from Dowd *et al.*, 2008) were used to amplify the V4–V6 region of bacterial 16S rDNA and the fungi-specific primers ITS1/ITS4 (White *et al.*, 1990) were used to amplify the ITS1, 5.8S rDNA and ITS2 regions of fungal rDNA. Primers *cbhI*F and *cbhI*R (Edwards *et al.*, 2008) were used to amplify a partial sequence of fungal cellobiohydrolase I. Primers for tag-encoded 454-Titanium pyrosequencing contained in addition sample tags separated from primers by spacers and Titanium A or B adaptors (Roche, Basel, Switzerland). Spacer sequences were designed to contain a trinucleotide, absent in all GenBank sequences at this position to avoid preferential amplification of some targets (Parameswaran *et al.*, 2007). Primer pairs were designed using OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html) and tested by cloning/sequencing. Tags and spacer sequences of all composite primers used for tag-encoded amplicon pyrosequencing in this study can be found in Supplementary Information.

PCR amplifications were performed in two steps. In the first step, each of three independent 50 μl reactions per DNA/cDNA sample contained 5 μl of 10 × polymerase buffer, 3 μl of 10 mg ml^{-1} bovine serum albumin, 2 μl of each primer (0.01 mM), 1 μl of PCR Nucleotide Mix (10 mM), 1.5 μl polymerase (2 U μl^{-1}; Pfu DNA polymerase:DyNAZyme II DNA polymerase, 1:24) and 2 μl of template DNA. Cycling conditions were 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 62 °C for 50 s, 72 °C for 30 s, followed by

72 °C for 10 min for primers eub530F/eub1100aR; 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 10 min for primers ITS1/ITS4; 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 51 °C for 45 s, 72 °C for 1 min 30 s, followed by 72 °C for 15 min for primers *cbhI*F/ *cbhI*R. Pooled PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). In all, 100 ng DNA was used as template in the second PCR performed under the same conditions except that fusion primers were used and cycle number was 10. PCR products were separated by electrophoresis and gel purified using the Wizard SV Gel and PCR Clean-Up System. DNA was quantified using ND1000 (NanoDrop, Wilmington, DE, USA), an equimolar mix of PCR products from all samples was made for each primer pair and the pooled products were mixed in a molar ratio of 12:4:1 (bacterial:fungal: *cbhI* amplicons). The mixture was subjected to sequencing on a GS FLX Titanium platform (Roche).

The pyrosequencing resulted in 329 820 reads of sufficient quality and a length >200 bases. Pyrosequencing noise reduction was performed using the Denoiser 0.851 (Reeder and Knight, 2010) and chimeric sequences were detected using UCHIME (Edgar, 2010) and deleted. In fungal community analyses, sequences >380 bases were used that contained the ITS1 region, 5.8S rDNA and a significant part of the ITS2 region. These sequences were truncated to 380 bases, clustered using CD-HIT (Li and Godzik, 2006) at 97% similarity (O'Brien *et al.*, 2005) to yield Operational Taxonomic Units (OTUs) and consensus sequences were constructed for all OTUs. PlutoF pipeline (Tedersoo *et al.*, 2010) was used to generate best species hits. In bacterial analysis, sequences of 350 bases were clustered at a 97% similarity and Ribosomal Database Project (Cole *et al.*, 2009) as well as BLASTn hits against GenBank were used to generate best hits (Altschul *et al.*, 1997). DNA/RNA ratio was calculated as sum of sequences derived from DNA divided by the sum of all sequences, and the L/H ratio was calculated similarly. Clusters of *cbhI* sequences were constructed using 400-base sequences at 96% similarity. Intron positions were recorded and introns removed from the DNA-derived sequences and DNA and cDNA-derived clusters were merged. For identification, *cbhI* sequences were retrieved from GenBank and also obtained by the analysis of isolates or cultured strains from the studied ecosystem by cloning/sequencing. Nucleotide sequences of OTUs with abundances over 0.3% were translated into amino-acid sequences in Bionumerics 7.0 (Applied Maths, Sint-Martens-Latem, Belgium).

Rarefaction and diversity analyses on OTUs/ clusters were performed at 8500 bacterial, 1000 fungal or 350 *cbhI* sequences per sample, to eliminate the effect of sampling effort and used for clustering as described above. Richness and diversity indices were calculated using EstimateS 8.00 (http://viceroy.eeb.uconn.edu/estimates) and quality of Chao1 estimates was evaluated according to Kemp and Aller (2004).

One-way analysis of variance (ANOVA) with the Fisher's LSD *post hoc* test was used to analyse the statistical differences among treatments. To analyse the differences in bacterial and fungal communities and the *cbhI* sequences, principal component analysis was run with abundance data of all OTUs or clusters with >0.3% abundance. PC1 and PC2 loads were subjected to ANOVA with the Fisher's LSD *post hoc* test. Differences at $P<0.05$ were regarded as statistically significant.

Results and discussion

Microbial communities in P. abies *topsoil are diverse and vertically stratified*

The topsoil of the *P. abies* forest was strongly acidic (pH 3.7–3.8) and consisted of a 1–4-cm-thick litter horizon (L) and a 2–4-cm-thick organic (humic) horizon (H). The horizons were significantly different with respect to organic matter and C and N contents, and decreasing nutrient availability was reflected by a decrease in both bacterial and fungal biomass contents with depth. The results of qPCR showed a decrease of fungal-to-bacterial rDNA copy number ratio from 1.11 in the L horizon to 0.30 in the H horizon. The cDNA contained 10^5–10^6 copies of the bacterial 16S rRNA gene per nanogram cDNA, but only 10^2–10^4 copies per nanogram cDNA of fungal ITS region (Figure 1). More rapid organic matter transformation occurred in the L horizon than in the H horizon, as documented by higher activities of several extracellular enzymes, especially those hydrolysing glucans (α- and β-glucosidase and cellobiohydrolase; Figure 1).

Bacterial communities analysed at 8500 sequences per sample showed about 1500 OTUs per sample in the L and H horizon-derived DNA (LD and HD) and in the L-derived RNA (LR) samples, about 1200 OTUs were identified in the H-derived RNA (HR). Also, the Chao1 estimator predictions were lower for HR. The RNA-derived communities were less diverse and less even than the DNA-derived communities, particularly in the H horizon (Supplementary Table 1). Principal component analysis followed by ANOVA showed significant differences in community composition among LD, HD, LR and HR ($P<0.00001$ for differences among L and H as well as among DNA/RNA).

In the DNA community, *Steroidobacter* (OTU4) was the most abundant in all L samples. In the H horizon, Gp1 Acidobacterium (OTU1) was the most abundant at three sites and Gp2 Acidobacterium (OTU5) at one site (Supplementary Table 2; Figure 1). Members of 505 genera were found in the entire pooled community, with most of the sequences belonging to Gp1, Gp3 and Gp2 *Acidobacteria* (on average 17.2%, 11.2% and 8.4%,

respectively), the *Actinobacteria Actinoallomurus* (7.1%), *Conexibacter* (1.3%) and *Iamia* (1.1%), and *Proteobacteria Steroidobacter* (5.4%), *Rhodoplanes* (3.3%), *Phenylobacterium* (2.1%), *Desulfomonile* (1.7%) and *Burkholderia* (1.5%; Supplementary Table 2). Of the most abundant OTUs, 33 (*Chondromyces*), 53 and 20 (*Phenylobacterium*) and 39 (*Caulobacteraceae*) were identified as highly enriched in the RNA-derived community and several taxa showed preferential association with either the L or the H horizon (Figure 2; Supplementary Table 2).

Bacterial sequences belonged to 21 phyla, but only 8 were recorded with abundances over 0.1%. In both horizons, *Proteobacteria*, *Acidobacteria* and *Actinobacteria* were dominant, comprising 80–90% of all sequences; this dominance was even stronger in the RNA (Figure 3). In the L horizon, the RNA community was enriched in *Acidobacteria* and *Firmicutes*, while most of the minor phyla were less represented. In the H horizon, *Actinobacteria* were more abundant in the RNA community (Supplementary Table 2; Supplementary Figure 1).

Bacterial abundance and diversity have been reported to decrease with decreasing soil pH (Lauber *et al.*, 2009; Rousk *et al.*, 2010). Despite this, a highly diverse bacterial community was found in our strongly acidic soil. Compared with other soils with pH <4, in which a high degree of dominance by *Acidobacteria*, around 63%, was previously reported (Lauber *et al.*, 2009), the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Verrucomicrobia* were more represented in the *P. abies* forest. The bacterial community in the soil of the study area is specific in several aspects. The genus *Chitinophaga*, which was abundant in a previous study that compared different soils (Fulthorpe *et al.*, 2008), was found at a frequency of only 0.1% in this study; the genera *Actinoallomurus* and *Steroidobacter*, ranked among the five most abundant genera in the *P. abies* forest, were not recovered in the previous study.

Deep sequencing analyses of bacterial communities associated with litter have not previously been reported. Here, we show that the litter horizon exhibited higher phylogenetic diversity and a

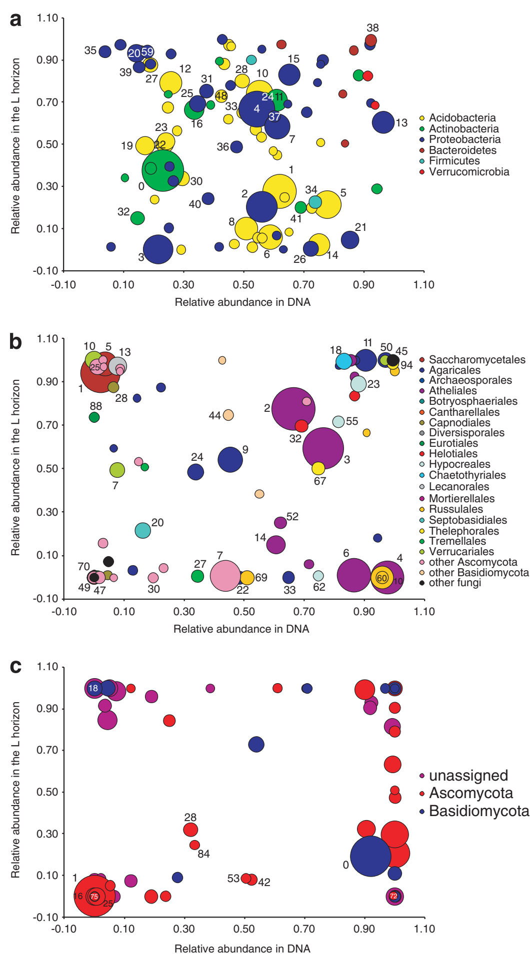

Figure 2 Distribution of major bacterial and fungal OTUs and *cbhI* clusters from *Picea abies* forest topsoil between the L and H horizons and between DNA and RNA. The data represent mean values from four sampling sites. Symbol areas correspond to relative abundance in the combined set of DNA and RNA sequences from both horizons. (**a**) Bacteria, identifications: 0 = *Actinoallomurus*; 1 = Gp1 Acidobacterium; 2 = *Rhodoplanes*; 3 = Rhodospirillales; 4 = *Steroidobacter*; 5 = Gp2 Acidobacterium; 6 = Gp1 Acidobacterium; 7 = *Rhizobiales*; 8 = Gp2 Acidobacterium; 10 = Gp1 Acidobacterium; 11 = Frankineae; 12 = Gp3 Acidobacterium; 13 = *Afipia*; 14 = Gp2 Acidobacterium; 15 = *Burkholderia*; 16 = Actinomycetales; 19 = Gp3 Acidobacterium; 20 = *Phenylobacterium*; 21 = *Desulfomonile*; 22 = Gp3 Acidobacterium; 23 = Gp3 Acidobacterium; 24 = *Ferrithrix*; 25 = Acetobacteraceae; 26 = Rhizobiales; 27 = Gp3 Acidobacterium; 28 = Gp1 Acidobacterium; 30 = Gp3 Acidobacterium; 31 = *Acidisphaera*; 32 = *Actinoallomurus*; 33 = Gp1 Acidobacterium; 34 = *Sporomusa*; 35 = *Chondromyces*; 36 = Acetobacteraceae; 37 = *Steroidobacter*; 38 = Chitinophagaceae; 39 = Caulobacteraceae; 40 = Rhizobiales; 41 = *Mycobacterium*; 48 = Gp1 Acidobacterium; 59 = *Phenylobacterium*. (**b**) Fungi, OTU identifications: 1 = Ascomycete; 2 = *Tylospora fibrillosa*; 3 = *Piloderma*; 4 = *Piloderma*; 5 = Ascomycete; 6 = *Tylospora asterophora*; 7 = *Cenococcum geophilum*; 8 = *Verrucaria*; 9 = *Hygrophorus olivaceoalbus*; 10 = *Russula cyanoxantha*; 11 = *Cortinarius biformis*; 13 = *Lecanora*; 14 = *Tylospora fibrillosa*; 18 = *Cladophialophora minutissima*; 20 = *Auriculoscypha*; 22 = *Inocybe*; 23 = Ascomycete; 24 = Basidiomycete; 25 = Ascomycete; 27 = *Cryptococcus podzolicus*; 28 = *Mycocentrospora acerina*; 29 = Ascomycete; 30 = Ascomycete; 32 = *Meliniomyces vraolstadiae*; 33 = *Amanita spissa*; 44 = *Phellopilus*; 45 = Chytridiomycete; 47 = *Alternaria alternata*; 49 = *Cenococcum geophilum*; 50 = *Cortinarius gentilis*; 52 = *Piloderma*; 55 = *Neofusicoccum*; 60 = *Russula cyanoxantha*; 62 = *Trichosporon porosum*; 67 = *Pseudotomentella*; 69 = *Russula cyanoxantha*; 70 = *Mycoarthris*; 88 = *Elaphocordyceps*; 94 = *Tomentella sublilacina*. (**c**) *cbhI*, clusters with sequence similarities to genes of known *cbhI* producers: 0 = *Mycena*; 1 = *Xylariales* spp.; 16 = *Phacidium*; 18 = *Mycena*; 25 = *Phacidium*; 28 = *Xylariales* spp.; 42 = *Phialophora*; 53 = *Xylariales* spp.; 72 = *Phialophora*; 75 = *Ceuthospora* and 84 = *Phialophora* sp.

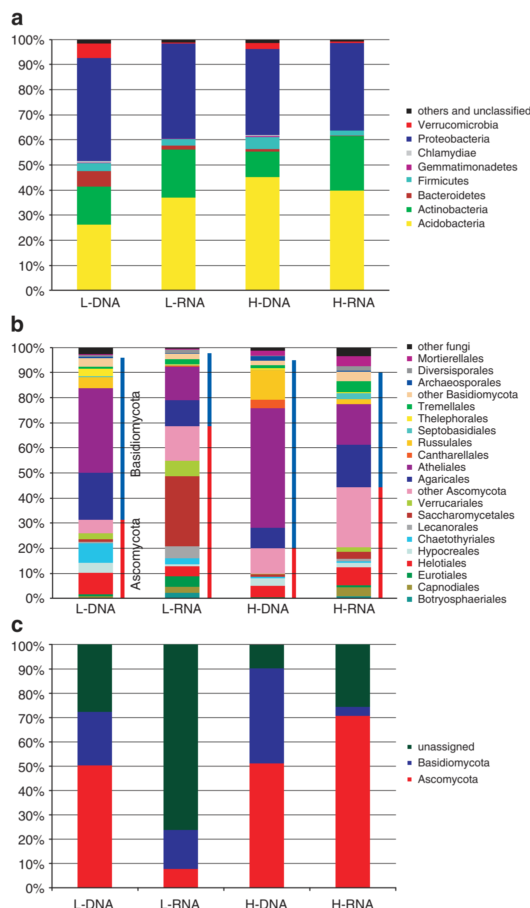

Figure 3 Phylogenetic assignment of bacterial, fungal and *cbhI* sequences from *Picea abies* forest topsoil. The data represent mean values from four study sites. (**a**) Bacteria, (**b**) fungi and (**c**) *cbhI* sequences.

reduced proportion of *Acidobacteria* in the total community. In the most abundant phylum, *Acidobacteria*, the members of Gp1, Gp2 and Gp3 detected were also reported to occur in other low pH soils (Jones *et al.*, 2009). Genome sequencing of Gp1 and Gp3 *Acidobacteria* and isolate culturing showed that these bacteria are able to decompose a variety of polysaccharides, including cellulose, xylan and chitin, and thus may be involved in decomposition (Ward *et al.*, 2009). Indeed, in our study, the Gp3 *Acidobacteria* were preferentially detected in the RNA, which indicates their activity during the decomposition period. The ecology of the other most abundant bacterial genera is unclear. Members of the genus *Actinoallomurus* (formerly belonging to *Actinomadura*) were repeatedly isolated from soils or litter, and some species from this genus are root endophytes. The genus *Phenylobacterium* contains bacteria from upper aerobic soil horizons capable of phenolic compound degradation.

Fungal communities sampled at 1000 randomly selected sequences per sample had between 150 and 220 OTUs with no significant differences between the DNA and RNA samples. The Chao1 estimates predicted higher diversity in LD and HD than in LR and HR (Supplementary Table 1). Principal component analysis followed by ANOVA showed significant differences in community composition among LD, HD, LR and HR ($P<0.0005$ for differences among L and H and $P<0.044$ for DNA/RNA).

In the DNA community, *Piloderma* sp. (OTU3) was dominant at two sites and *Tylospora fibrillosa* (OTU2) and *Cortinarius biformis* (OTU11) were each dominant at one site in the L horizon. In the H horizon, each of the sites was dominated by a different OTU (*Tylospora fibrillosa*, OTU2; *Tylospora asterophora,* OTU6; *Russula cyanoxantha*, OTU10; and *Piloderma* sp., OTU4). OTUs with the closest similarity to 422 different genera were recorded, the most abundant being *Tylospora* (14.8% of all sequences), *Piloderma* (12.8%), *Russula* (4.4%), *Cenococcum* (4.2%), *Cortinarius* (3.9%), *Hygrophorus* (2.9%), *Cladophialophora* (2.4%), *Amanita* (1.8%), *Cadophora* (1.7%), *Mortierella* (1.6%) and *Verrucaria* (1.6%; Supplementary Table 3). The distribution of the abundant OTUs among the L and H horizons and the DNA and RNA communities shows a strict confinement of many OTUs to either the L or the H horizon (Figure 2; Supplementary Table 3). Several of the most abundant OTUs were highly enriched in the RNA community (Supplementary Table 3).

Fungal sequences belonged mainly to Dikarya (53.5% *Basidiomycota* and 41.1% *Ascomycota*). *Glomeromycota* were represented by 2.24% of the sequences, *Mucoromycotina* by 1.77%, and *Chytridiomycota* by 0.73 (Supplementary Table 3). For several groups of phylogenetically related OTUs abundant in the ecosystem, no close sequence of an isolated strain was available. Some of these, including also the putative members of basal fungal lineages, exhibited high abundance in the RNA samples (Supplementary Figure 2). Members of the orders *Atheliales*, *Agaricales*, *Helotiales*, *Chaetothyriales* and *Russulales* were most abundant in the soil DNA; several minor orders, including *Botryosphaeriales*, *Lecanorales* and *Eurotiales* in the L horizon and *Tremellales* and *Capnodiales* in the H horizon, were infrequent in the DNA communities but highly abundant among the RNA sequences (Figure 3).

The composition of fungal communities has been previously shown to differ substantially between litter and organic horizons, while deeper soil horizons showed greater similarity (O'Brien *et al.*, 2005; Lindahl *et al.*, 2007). In several forest types, this is due to the higher abundance of saprotrophic fungi in litter and the dominance of ectomycorrhizal species

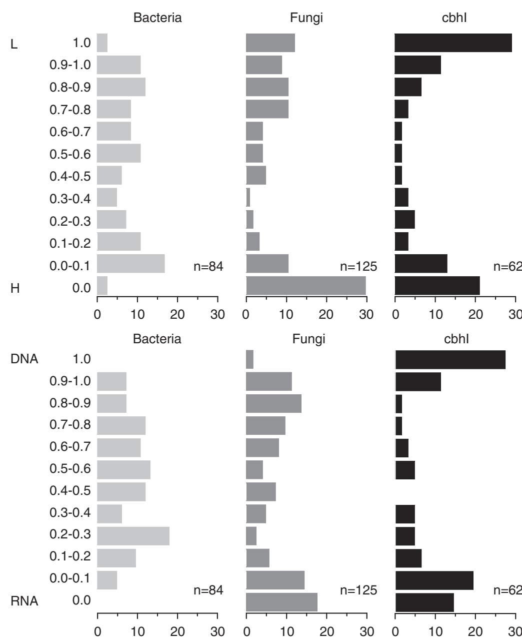

Figure 4 Distribution of bacterial and fungal OTUs and *cbhI* clusters from *Picea abies* forest soil among the L and H horizons and among DNA and RNA, in percents. Based on the data for OTUs/clusters with abundance >0.1%. The *y* axis represents relative share of transcripts in the L horizon or in the DNA. The values 0.0 and 1.0 represent OTUs/clusters present only in either the H or L horizon, in RNA or in the DNA.

in deeper soil (Lindahl *et al.*, 2007; Edwards and Zak, 2010). Although our results showed that ectomycorrhizal fungi were highly dominant in both horizons (most likely due to the shallow rooting of *P. abies*), we also confirmed profound differences between the two horizons; 42% of abundant species were only recovered from either the L or the H horizon (Figure 4). The most abundant genera of ectomycorrhizal fungi found in this study, *Piloderma* and *Tylospora* spp., are also the most abundant in the boreal *P. abies* forests in Finland and Sweden (Rosling *et al.*, 2003; Korkama *et al.*, 2006; Wallander *et al.*, 2010).

Ecological functions could be reliably assigned to 64–94% of the abundant members of the fungal community (Supplementary Table 3). Among these, ectomycorrhizal fungi dominated all communities, representing 83% of the sequences in LD, 95% in HD, 66% in LR and 69% in HR (Supplementary Figure 2C). During a period when decomposition processes prevail, a substantial reduction in the activity of ectomycorrhizal fungi compared with saprotrophs is expected (Yarwood *et al.*, 2009; Lindahl *et al.*, 2010). Saprotrophic and parasitic species were indeed significantly more represented in the RNA communities, the ratio of mycorrhizal/saprotrophic + parasitic fungi being 6.9 and 2.9 in the LD and LR and 21.2 and 2.3 in the HD and HR, respectively. Notably, we found many fungal sequences belonging to lichen-forming fungi and detected these preferentially in the RNA community (9.3% in LR).

Ecology of soil bacteria and fungi is largely different

In agreement with previous studies on the vertical stratification of soil decomposition processes (Wittmann *et al.*, 2004; Šnajdr *et al.*, 2008), we show that the L and H horizons differ significantly in both the total and relative amounts of bacterial and fungal biomasses (Figure 1). According to the phospholipid fatty acid/biomass C conversion factors (Anderson and Parkin 2007), the L horizon contained 2.9 times more fungal than bacterial biomass (10.7 and 3.7 mg biomass C per gram, respectively). In the H horizon, fungal and bacterial biomasses were equal (1.50 and 1.58 mg g^{-1}). The diversity of bacterial populations was considerably higher than that of fungal populations. When 1000 randomly selected sequences were analysed, 302–366 OTUs were found in bacteria, compared with 141–236 in fungi.

While the diversity estimates for DNA- and RNA-derived communities of bacteria were similar, the Chao1 estimators for fungal communities surprisingly showed that a more diverse community is revealed when RNA is analysed (Supplementary Table 1). The DNA- and RNA-derived communities of bacteria largely overlapped, and among the abundant OTUs none was found exclusively either in DNA or RNA. By contrast, 18% of fungal OTUs were found only in the RNA community, and 2% were found exclusively in the DNA community. Among *cbhI* sequences, indicating the presence of cellulolytic members of the fungal community, 27% were found only in DNA and 15% only in RNA (Figure 4). These data show that the DNA sequencing approaches miss a significant and functionally relevant part of microbial communities and our current knowledge largely based on this approach is incomplete. The high RNA/DNA ratios for some microbial taxa show that species with low abundance can be highly active.

Abundances of the major bacterial and fungal OTUs in different soils have been reported to be 2–3% and 7–17%, respectively (Fierer *et al.*, 2007; Buée *et al.*, 2009). In this study, the first to report on bacteria and fungi from the same soil, bacterial communities also showed higher evenness than fungal communities. The most abundant bacterial OTU accounted for 5–7% of all sequences, while the dominant fungal OTU in our ectomycorrhiza-dominated ecosystem represented up to >30% of all sequences. Between 30 and 60 of the most abundant bacterial and 6 and 22 of the most abundant fungal OTUs made up 50% of their respective communities (Supplementary Table 1).

Most bacterial OTUs with abundance >0.3% were recovered from all study sites. In contrast,

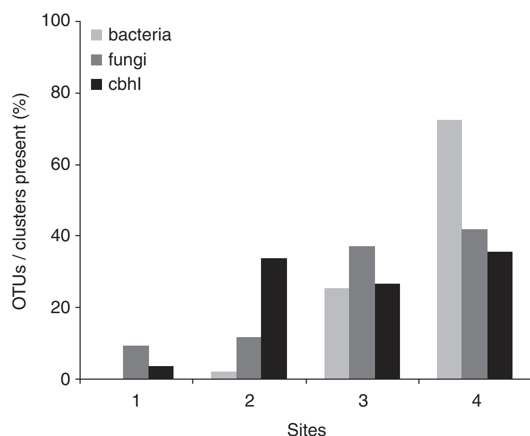

Figure 5 Number of sites in the *Picea abies* forest soil where DNA of dominant bacterial and fungal OTUs and *cbhI* clusters were detected. Only OTUs/clusters with abundance >0.3% in the DNA were considered; $n=50$ for bacteria, $n=54$ for fungi and $n=56$ for *cbhI*. To correct for the same sampling depth, 350 sequences of each target were randomly selected from each sample.

majority of fungal OTUs and *cbhI* clusters were found only at one to three sites (Figure 5). Also, the abundance of bacteria across study sites varied considerably less than that of fungi; the mean coefficient of variation was 0.56 for bacteria and 1.30 for fungi. This applied even in the case of the most abundant fungal taxa, for example, the second-most abundant fungal OTU was absent at one study site. The uneven spatial distribution of fungi in the ecosystem is likely a consequence of a combination of factors including the size of organisms, mobility and the association of many taxa with large nutrient patches or plant roots. This is supported by the fact that fungi forming large mycelial systems (for example, *Russulales* and *Agaricales*) showed higher variation in abundance than species with limited mycelia (for example, *Mortierellales* and *Archaeosporales*).

The affinity of microbial taxa and *cbhI* clusters for either the L or H horizon and their differential abundance in DNA versus RNA points to their different niches and ecological roles (Supplementary Tables 2–4). Over 60% of dominant fungal OTUs and as much as 74% of *cbhI* clusters showed 10-fold enrichment in either the L or H horizons. In contrast, vertical stratification was less distinct in bacterial OTUs (Figure 4). This observation further stresses the importance of fungi in shaping the spatial structure of the forest floor.

Cellobiohydrolase genes exhibit high diversity in the soil metagenome and metatranscriptome

Total RNA extracted from soils contains <10% mRNA (Urich *et al.*, 2008); even after mRNA enrichment it still contains both rRNA and transcripts of abundant genes such as those encoding ribosomal proteins (Bailly *et al.*, 2007). This leads to low recovery of targeted sequences of functional genes by shotgun sequencing approaches. For example, only nine gene clusters of denitrification genes were obtained from 77 000 metagenome-derived clones (Demaneche *et al.*, 2009). Amplification of target sequences is thus the only way to assess the diversity of functional genes.

Here, we show for the first time that a single eukaryotic functional gene can be analysed at a depth that allows diversity estimation; a reliable Chao1 diversity prediction of 46 ± 9 *cbhI* clusters per sample was obtained for the HR sample. When samples from all sites were analysed together, a total of 456 clusters were predicted for LD, 344 for HD, 201 for LR and 99 for HR, with sufficient sampling effort for LR and HR. Approximately 40% and 25% of sequences present in the DNA were being transcribed in the L and H horizons, respectively. Because most of the analysed fungi harbour more than one *cbhI* gene (Baldrian and Valášková, 2008; Edwards *et al.*, 2008), the diversity of cellulolytic fungi in forest topsoil can be estimated only roughly at 50–300. This means that a considerable proportion of the fungal community transcribes or at least harbours the *cbhI* gene. Almost all *cbhI* clusters showed distinct association with either the L or H horizons, indicating only a minor overlap of cellulolytic fungal communities between horizons. Principal component analysis followed by ANOVA showed significant differences in *cbhI* pool composition among LD, HD, LR and HR ($P<0.0019$ for differences among L and H and $P<0.0054$ for DNA/RNA).

Current attempts to assign fungal producers to the sequences of functional genes derived from soil metagenomes suffer from a lack of sequence information in public databases. Because of this, the closest hits for most *cbhI* clusters in public databases were rather distant, with only three clusters showing >96% similarity. By sequencing *cbhI* genes from fungi occurring in the soil, we were able to identify the taxonomic affiliation of producers for 13 additional clusters (Supplementary Table 4). Some of the most abundant *cbhI* sequences were transcribed by fungi with low abundances in the ecosystem (for example, *Mycena* sp. and *Xylariales*), showing the importance of low-abundance species for cellulose hydrolysis. Phylogeny trees constructed using cDNA and peptide sequences (Supplementary Figure 3) allowed coarse taxonomic placement of producers for 24–90% of dominant *cbhI* clusters into either *Ascomycota* or *Basidiomycota* (Figure 3). In the H horizon, where 75–90% of sequences were assigned, genes of both taxa were equally present, but the transcripts were mainly of ascomycetous origin (>95% of assigned sequences).

To match the sequences of genes and transcripts, introns were removed from DNA sequences (Supplementary Figure 3). There were 28 DNA clusters

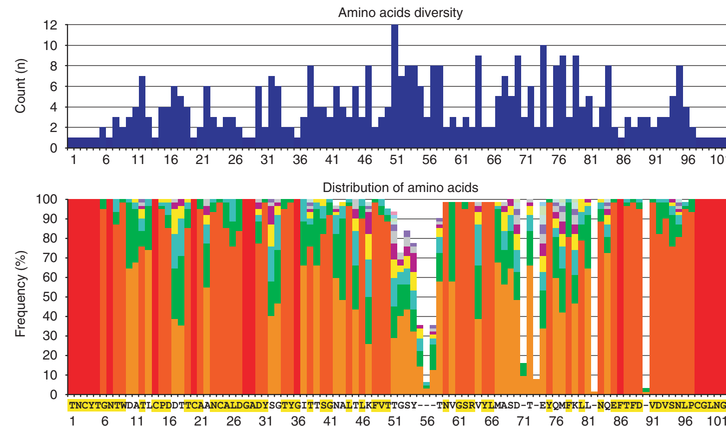

Figure 6 Diversity of amino-acid composition and frequency of alternative amino acids over the length of a *cbhI* internal peptide obtained by translation of the 62 most abundant *cbhI* sequences detected in *Picea abies* forest soil. Abundance of alternative amino acids at each position is colour coded; the identity of the most abundant amino acid at each position is indicated, and consensus amino acids (>75%) are highlighted.

containing one intron and one containing four introns. The internal peptide of *cbhI* obtained after intron removal varied in length and contained both conserved and highly variable regions (Figure 6). The consensus sequence derived from this study differed from the one based on published *cbhI* sequences from isolated fungal strains at 4 amino-acid positions out of 101 (Edwards *et al*., 2008). Thus, the depth of environmental amplicon sequencing may contribute to the construction and evaluation of better primers and qPCR probes for targeted functional genes.

Conclusions

Much of what is currently known about the ecology of soil microbial communities has been inferred from studies targeting DNA. Despite similar diversity of microbial communities based on DNA and RNA analysis, the fact that several major fungal OTUs were found exclusively in the RNA pool and that several active bacterial OTUs exhibited low abundance in the DNA pool demonstrates the limitations of DNA-based surveys, which likely miss considerable portions of active microbial populations. In the soil ecosystem, bacterial and fungal communities differ in their spatial distributions with fungal taxa more distinctly confined to either the litter or the organic horizon of soil and more heterogeneously distributed in the ecosystem. The diversity and distribution of functional genes responsible for important biogeochemical processes and consequently of their producers can be efficiently targeted by amplicon sequencing. Low abundance of several fungal taxa highly expressing the *cbhI* gene suggests that these species are highly important for decomposition.

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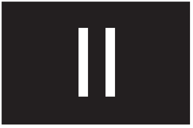

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ORIGINAL ARTICLE

Fungal community on decomposing leaf litter undergoes rapid successional changes

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Fungi are considered the primary decomposers of dead plant biomass in terrestrial ecosystems. However, current knowledge regarding the successive changes in fungal communities during litter decomposition is limited. Here we explored the development of the fungal community over 24 months of litter decomposition in a temperate forest with dominant *Quercus petraea* using 454-pyrosequencing of the fungal internal transcribed spacer (ITS) region and cellobiohydrolase I (*cbhI*) genes, which encode exocellulases, to specifically address cellulose decomposers. To quantify the involvement of phyllosphere fungi in litter decomposition, the fungal communities in live leaves and leaves immediately before abscission were also analysed. The results showed rapid succession of fungi with dramatic changes in the composition of the fungal community. Furthermore, most of the abundant taxa only temporarily dominated in the substrate. Fungal diversity was lowest at leaf senescence, increased until month 4 and did not significantly change during subsequent decomposition. Highly diverse community of phyllosphere fungi inhabits live oak leaves 2 months before abscission, and these phyllosphere taxa comprise a significant share of the fungal community during early decomposition up to the fourth month. Sequences assigned to the *Ascomycota* showed highest relative abundances in live leaves and during the early stages of decomposition. In contrast, the relative abundance of sequences assigned to the *Basidiomycota* phylum, particularly basidiomycetous yeasts, increased with time. Although cellulose was available in the litter during all stages of decomposition, the community of cellulolytic fungi changed substantially over time. The results indicate that litter decomposition is a highly complex process mediated by various fungal taxa.
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Keywords: fungi; litter decomposition; cellulose; endophyte; temperate forests

Introduction

Plant litter represents a major source of organic carbon in forest soils. Its decomposition is a complex process that involves mineralisation and transformation of organic matter. Decomposition of plant litter is a key step in nutrient recycling (Berg *et al.*, 2001). As most of the plant biomass-derived carbon in the temperate and boreal forests is mineralised in the litter layer, an understanding of this process and the microorganisms involved is essential for the identification of factors that affect global carbon fluxes.

Fungi are considered to be the key players in litter decomposition because of their ability to produce a wide range of extracellular enzymes, which allows them to efficiently attack the recalcitrant lignocellulose matrix that other organisms are unable to decompose (Kjoller and Struwe, 1982; de Boer *et al.*, 2005). Biochemical decomposition of leaf litter is a sequential process that initially involves the loss of the less recalcitrant components (for example, oligosaccharides, organic acids, hemicellulose and cellulose) followed by the degradation of the remaining highly recalcitrant compounds (for example, lignin or suberin). Litter quality changes during the course of its transformation and so does the activity of litter-associated microorganisms (Dilly *et al.*, 2001). These changes are accompanied by a succession of microbial litter decomposers that reflect the varied catabolic capabilities that are sequentially required to complete the process of litter decomposition (Frankland, 1998; Osono *et al.*, 2006).

The ability of fungi to decompose leaf litter has been investigated many times under laboratory conditions (for example, Osono, 2007; Baldrian *et al.*, 2011). Furthermore, many studies have combined litterbag techniques with cultivation-based methods followed by the isolation and identification of fungal decomposers (Koide *et al.*, 2005; Osono, 2005; Zhang *et al.*, 2008; Osono *et al.*,

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2009). Using these methods, fungi involved in the decomposition of litter have been divided into early, intermediate and late decomposers (Frankland, 1998; Tang *et al.*, 2005). This observation was supported by a recent study performed by Šnajdr *et al.* (2011), as these three phases were distinguished during oak litter decomposition based on the differences in the activity of extracellular enzymes and the rates of decomposition of the individual litter constituents. In most previous studies, fungi from the *Ascomycota* phylum were found to dominate during the initial stages of litter decay along with a few basidiomycetous fungi. The abundance of fungi from the *Ascomycota* phylum decreases during the process of degradation as they are gradually replaced by fungi from the *Basidiomycota* phylum, especially the saprotrophic cord formers, during the later stages of decomposition (Frankland, 1998; Osono, 2007).

Plant organic matter transformation leads to the disappearance of easily utilisable compounds and to the formation of recalcitrant ones. As a consequence, the chemical and spatial heterogeneity of the substrate changes with time. This process can theoretically result in the formation of novel niches and a potential increase in fungal diversity or to the creation of more uniform environment with a potential decrease in diversity. Both scenarios have been reported from litter or wood (Melillo *et al.*, 1989; Dickie *et al.*, 2012) but the actual development of fungal community on decaying litter is so far unknown. Culture-dependent approaches are typically selective because only a small fraction of microbial taxa grow under the conditions used for strain isolation (Amann *et al.*, 1995). Molecular methods, such as community fingerprinting or direct sequencing of cloned PCR sequences that have recently been applied to litter (Aneja *et al.*, 2006; Kubartova *et al.*, 2009) suffered from limited resolution. Therefore, next-generation sequencing approaches currently represent the only technique that can be used to sufficiently describe the development of fungal community composition during succession.

The degradation of plant leaves is not limited to the litter layer on the forest floor. Indeed, the decomposition process begins as soon as the leaf is formed (Stone, 1987). Phyllosphere fungi that are established in the interior or on the surfaces of live leaves have the advantage of gaining access to readily available nutrients in live leaves and later, after senescence, to the dead leave biomass. Recently, 454-pyrosequencing was used to assess fungal diversity in live oak leaves and demonstrated the presence of a diverse fungal community (Jumpponen and Jones, 2009a, b). It is highly probable that at least some of these fungi participate in litter decomposition. There is some evidence that certain phyllosphere fungi are able to transform various components of litter because they produce the extracellular enzymes that are involved in decomposition in pure culture and their ability to decompose litter material has been described (Korkama-Rajala *et al.*, 2008; Žifčáková *et al.*, 2011). Although potential leaf endophytes have been isolated from litter in various stages of decomposition (Osono, 2002; Koide *et al.*, 2005), their importance in the community of litter-associated fungi is currently unknown.

The aim of this study was to characterise the development of the fungal community composition over 24 months following litterfall in a temperate forest dominated by *Quercus petraea*. As some litter components, including cellulose, remain present in a considerable quantity during the entire 24-month period (Šnajdr *et al.*, 2011), the fungi capable of cellulose decomposition may be present during all phases of decomposition. To address this possibility, the composition of the gene pool of the *cbhI* exocellulase gene, which is an enzyme that catalyses the rate-limiting step in the decomposition of cellulose (Baldrian and Valášková, 2008; Edwards *et al.*, 2008), was monitored at various stages of litter decomposition. To evaluate the role of phyllosphere fungi in litter decomposition, fungal communities associated with live *Q. petraea* leaves and senescent leaves immediately before abscission were also analysed. The results of this study are discussed in light of previously published data derived from the same experiment where litter decomposition (mass loss), fungal and bacterial biomass content based on ergosterol and phospholipid fatty acid analysis, and the activity of the extracellular enzymes in the litterbags were explored by Šnajdr *et al.* (2011).

Materials and methods

Study site and sample collection

The study site was an oak (*Q. petraea*) forest in the Xaverovský Háj Natural Reserve, near Prague, Czech Republic (50°5′38″N, 14°36′48″E). The site was previously explored with respect to the activity of decomposition-related extracellular enzymes in the forest topsoil (Šnajdr *et al.*, 2008) and during the successive transformation of *Q. petraea* litter (Šnajdr *et al.*, 2011). In this study site, the saprotrophic fungi were characterised (Valášková *et al.*, 2007; Baldrian *et al.*, 2011). The soil was acidic cambisol with a litter thickness of 0.5–1.5 cm, a pH of 4.3, a C content of 46.2% and an N content of 1.76%. The mean annual temperature at the soil surface was 9.3 °C (winter mean 1.3 °C, summer mean 16.6 °C; Baldrian *et al.*, in press).

The litterbag experiment was run as described previously (Šnajdr *et al.*, 2011). Litter material (*Q. petraea* leaves, tree age 100–120 years) for litterbag construction was collected immediately after abscission and allowed to air dry at 20 °C. Litterbags containing 5 g of air-dried leaves (10 × 20 cm, 1 mm nylon mesh size) were placed on the top of the litter horizon at the study site at the end of the litterfall

season. To prevent extensive desiccation, litterbags were overlaid with freshly fallen oak leaves. Litterbags were removed after 2, 4, 8, 12 and 24 months of incubation, three litterbags were collected at each sampling time for DNA extraction. For the analysis of the phyllosphere fungal community composition, live *Q. petraea* leaves were collected 2 months before abscission (August) by hand-picking (month −2), and senescent leaves were collected during the litterfall period by gently shaking oak twigs and collecting falling leaves before their contact with the soil (month 0). Collected material was transferred to the laboratory and processed immediately. Leaves or litter were cut into 0.25 cm^2 pieces and used immediately for DNA extraction. The same material was also used for chemical analyses, measurement of enzyme activities and quantification of microbial biomass as described in Šnajdr *et al.* (2011).

*454-Pyrosequencing of fungal internal transcribed spacer (ITS) and cellobiohydrolase I (*cbhI*) genes*

The total genomic DNA was extracted from 300 mg of material using the Powersoil Kit (MoBio, Carlsbad, CA, USA). The primers ITS1/ITS4 (White *et al.*, 1990) were used to amplify the ITS1 region, the 5.8S ribosomal DNA and the ITS2 region of the fungal ribosomal DNA. The primers *cbhI*F and *cbhI*R (Edwards *et al.*, 2008) were used to amplify a partial sequence of the fungal *cbhI* gene. These primers amplify *cbhI* genes belonging to the GH7 family of fungi from the *Basidiomycota*, *Ascomycota* and *Mucoromycotina* unless the template contains intron in the primer sequence (Štursová *et al.*, 2012).

A two-step PCR amplification using composite primers containing multiplex identifiers was performed to obtain amplicon libraries for 454-pyrosequencing following a previously described method (Baldrian *et al.*, 2012). PCR amplicons were quantified using the Quant-iT PicoGreen Kit (Invitrogen, Grand Island, NY, USA). An equimolar mix of PCR products was prepared for each primer pair, and the pooled products were sequenced on a GS FLX Titanium platform (Roche, Basel, Switzerland). Fungal ITS sequences were analysed from all sampling times, and the *cbhI* gene diversity was analysed in the samples collected at −2, 0, 4 and 12 months.

Bioinformatic analysis

The pyrosequencing data were processed as described previously (Baldrian *et al.*, 2012). Pyrosequencing noise reduction was performed using the Denoiser 0.851 (Reeder and Knight, 2010) and chimeric sequences were detected using UCHIME (Edgar, 2010) and deleted. Fungal sequences were shortened to 380 bases and clustered using cd-hit (Li and Godzik, 2006) at a 97% similarity level (O'Brien *et al.*, 2005) to obtain the operational taxonomical units (OTUs). Consensus sequences were constructed for each cluster, and the closest hits were identified using the PlutoF pipeline (Tedersoo *et al.*, 2010). For the *cbhI* gene, the sequences were truncated to 300 bp and clustered at a 96% similarity level (Baldrian *et al.*, 2012) to obtain the OTUs. Consensus sequences were constructed, and the introns were removed. Data sets containing the *cbhI* sequences representing the OTUs and sequences retrieved from GenBank were aligned using SeaView 4 (http://pbil.univ-lyon1.fr/software/seaview.html) with MUSCLE (http://www.drive5.com/muscle/). Maximum likelihood phylogenetic trees were computed with the GTR substitutions model using GARLI (http://www.molecularevolution.org/sofware/phylogenetics/garli/garli_create_job). The OTUs of *cbhI* genes that clustered with sequences of known fungal taxa from the GenBank with bootstrap support >70% were taxonomically assigned to fungal phyla.

Sequence data have been deposited in the MG-RAST public database (http://metagenomics.anl.gov/, data set numbers 4497081.3 for fungal ITS region and 4497080.3 for *cbhI* genes).

Diversity and statistical analyses

Owing to the fact that the sampling depth achieved in this study did not allow to make realistic estimates of total diversity and since next-generation sequencing derived data were demonstrated to be affected by artefacts (Tedersoo *et al.*, 2010), the only measure of diversity of OTUs used was the amount of the most abundant OTUs that represented 80% of all sequences. This metric in our opinion fairly represents the diversity of the quantitatively important part of the fungal or *cbhI* community. To avoid possible effects of variable sampling depth, these estimates were calculated for a data set containing 700 randomly chosen ITS sequences or 495 *cbhI* sequences from each litterbag. The sequences were clustered again as described above. The OTU richness and Chao1 were calculated using EstimateS 8.00 (http://viceroy.eeb.uconn.edu/estimates).

One-way analysis of variance with the Fisher's least significant difference *post hoc* test was used to analyse the significant differences in relative abundance of individual OTUs or fungal taxa among sampling times. Principal component analysis was performed with the relative abundance data of the 50 most abundant fungal genera. PC1 and PC2 loads were subjected to analysis of variance with the Fisher's least significant difference *post hoc* test. Differences with a $P<0.05$ were regarded as statistically significant.

Results

Fungal communities associated with oak leaves

In total, 23 760 sequences of the fungal ITS region with >380 bp were used for analysis after denoising and removal of the chimeric sequences and

sequences not belonging to fungi (<0.6%). These sequences clustered into 1874 OTUs (including 1193 singletons) at a 97% similarity level. Although 80% of all sequences at month −2 were represented by 23 dominant OTUs, at month 0 70–90% of sequences belonged to the single most abundant OTU (assigned to *Mycosphaerella punctiformis*). Within a relatively short time (month 4), the diversity peaked with 30 OTUs representing 80% of the total fungal community at month 8 and then levelled off (Figure 1).

A total of 387 fungal genera were identified as the closest hits of individual OTUs. *Mycosphaerella*, *Naevala*, *Troposporella* and *Trichosporon* were the most abundant fungi in the amplicon pool. The 50 most abundant fungal OTUs with their closest identified hits and abundances are listed in Supplementary Table 1. In all, 40 of the 50 most abundant fungal OTUs and 27 of the top 33 genera demonstrated significant changes in abundance over time (Supplementary Table 1, Figure 2). Altogether, the ascomycetous OTU 0 (closest hit: *Mycosphaerella punctiformis*) and OTU 1 (*Naevala minutissima*) were the most abundant fungi. OTU 0 was predominant during the early stages of succession (month −2 and 0) but disappeared almost completely during later stages. OTU 1 and OTU 4 (*Athelia*) were highly abundant during the initial stages of litter decomposition (months 2 and 4). However, OTU 3 and OTU 11 (both *Troposporella fumosa*) dominated the later stages of decomposition (months 8 and 12) but were almost absent in other samples. The latest stages of litter decomposition were dominated by OTU 2 (*Trichosporon porosum*) and OTU 5 (*Trichosporon miniliiforme*) (Supplementary Table 1).

The majority of fungal sequences were assigned to the *Ascomycota* (71%) and *Basidiomycota* (26%) phylum. *Glomeromycota* were represented by 1.8% of all sequences, and fungi from *Mucoromycotina* comprised 0.76% of all sequences. Fungi from the *Ascomycota* phylum dominated in the live and senescent leaves, which is in contrast to month 24 when fungi from the *Basidiomycota* phylum represented 60% of the amplicons. *Glomeromycota* and *Mucoromycotina* sequences were rare until month 4 and then rapidly increased until month 8 and 24 when they represented 8.6% and 2.2% of the amplicon pool, respectively (Figure 3). The most abundant fungal orders were *Capnodiales* (22% of sequences), *Helotiales* (20%) and *Tremellales* (12%). Members of the *Helotiales* order were present during

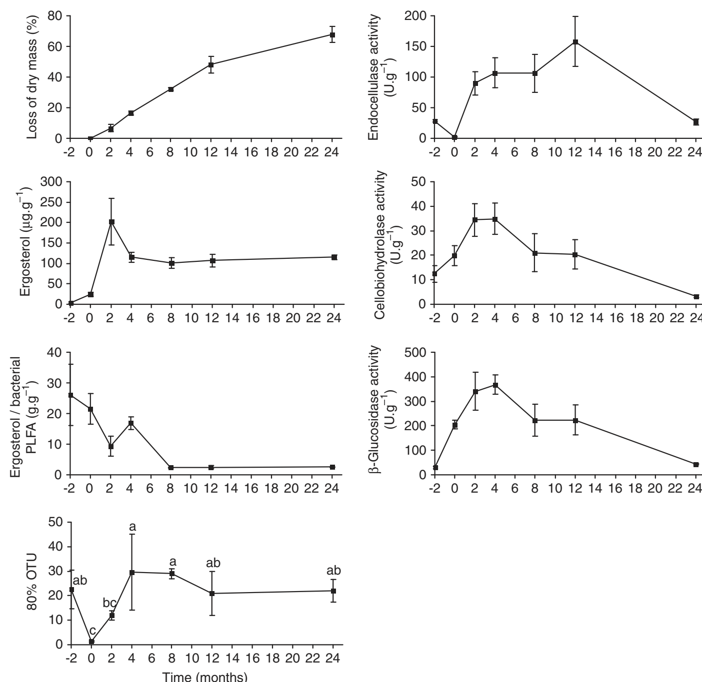

Figure 1 Loss of dry mass, activity of extracellular enzymes, development of fungal and bacterial biomass and estimates of fungal diversity in *Q. petraea* live leaves and leaves at different stages of decomposition. Fungal biomass is expressed as ergosterol content. The ratio of fungal and bacterial biomass is based on the ratio of ergosterol content and the content of bacteria-specific phospholipid fatty acids. Data on leaf chemistry and microbial biomass are derived from Šnajdr *et al.* (2011). 80% OTU represents the number of the most abundant OTUs, which represent 80% of all sequences. The data are shown as the means and s.e. from three litterbags.

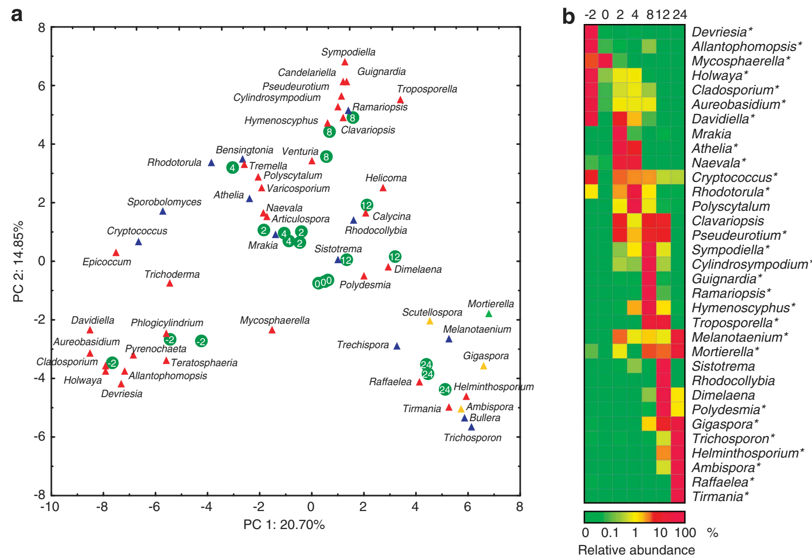

Figure 2 (**a**) Principal component analysis of the relative abundance of the 50 most abundant fungal genera in *Q. petraea* live leaves and leaves at different stages of decomposition. *Ascomycota*—red, *Basidiomycota*—blue, *Glomeromycota*—yellow, *Mucoromycotina*—green. Green circles with numbers indicate the positions of individual samples (litterbags) with ages in months. (**b**) Time course of the relative abundance of dominant fungal genera in *Q. petraea* live leaves and leaves at different stages of decomposition. Mean abundances are shown for each time point. A statistically significant effect of time on abundance is indicated by an asterisk ($P<0.05$, analysis of variance (ANOVA) followed by Fisher's *post hoc* test).

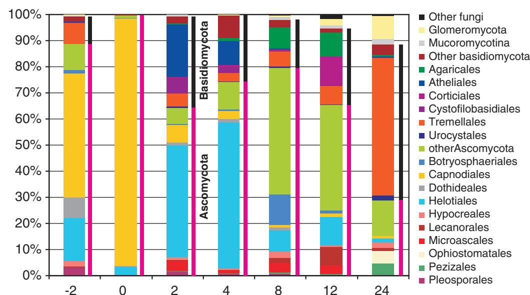

Figure 3 Phylogenetic assignment of fungal sequences from *Q. petraea* live leaves and leaves at different stages of decomposition. The data are represented as the mean values from three litterbags.

all phases of succession and peaked at month 2 and 4 together with fungi from the *Atheliales* family. Fungi belonging to the ascomycetous order *Capnodiales* dominated among amplicons from the live and senescent leaves, whereas the basidiomycetous order *Tremellales* predominated at later stages (Figure 3).

Each sampling time was characterised by a specific fungal community, which was different from the community in the previous or the next stage. When the abundance of the top 50 fungal genera was analysed by principal component analysis, the first two canonical axes explained 20.70% and 14.85% of the total variability (Figure 2). The analysis of variance of the PC1 and PC2 loadings demonstrated that community changes over time were significant between any two dates except between months 2 and 4 ($P<0.015$). This is supported by the fact that most of the fungi that were highly abundant at a certain time only dominated for a short period. Among the 28 genera that represented $>3\%$ of the sequences at any particular sampling time, 20 did not exceed 1% at any other time (Figure 2).

The amplicons from live *Q. petraea* leaves were dominated by the *Ascomycota* (88.5%) fungi, and *Capnodiales*, *Helotiales*, *Dothideales* and *Pleosporales* were the major orders. The *Basidiomycota* phylum (10.6%) was mainly represented by *Tremellales* (Figure 3). The fungal community on senescent leaves was dominated by the same OTUs that were dominant on live leaves, and the fungi that were highly abundant on live leaves comprised approximately 50% of all fungi until month 4 (Figure 4).

Cellulose-decomposing fungi associated with oak leaves

The gene *cbhI* encoding for cellobiohydrolase was used as a marker for the cellulolytic members of the fungal community. In total, 3351 denoised, non-

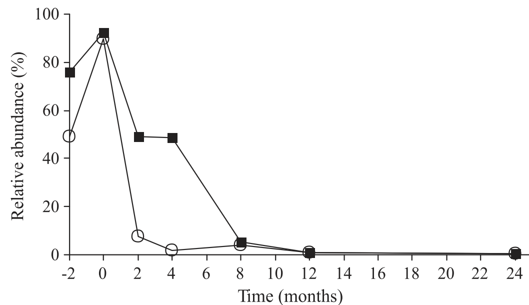

Figure 4 The persistence of fungal OTUs recorded in live *Q. petraea* leaves (endophytes) during subsequent decomposition of litter. Data represent the sum of the relative abundances of dominant endophytes with abundances >5% in live leaves (open circles) and abundant endophytes (>1%; black squares).

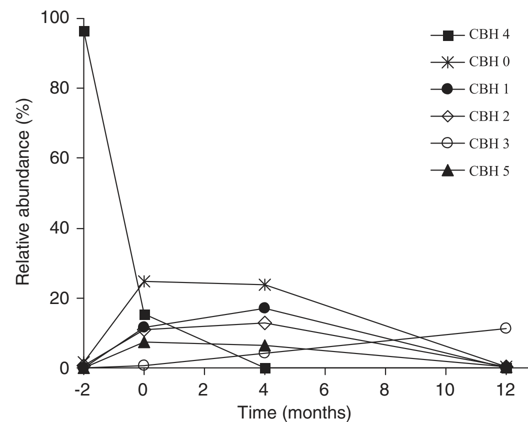

Figure 5 Time course of the relative abundance of dominant cellulolytic fungi represented by the *cbhI* gene OTUs in *Q. petraea* live leaves and leaves at different stages of decomposition. The data are shown as the mean values from three litterbags.

chimeric sequences of *cbhI* were analysed. The sequences clustered into 235 OTUs (including 107 singletons). The diversity of the cellulolytic fungal community increased between months 4 and 12: 80% of the *cbhI* gene sequences were represented by only 7 ± 1 and 8 ± 1 dominant OTUs during months 0 and 4 while it was 26 ± 2 at month 12.

The most abundant OTUs were OTU 4 and OTU 0, which represented 28% and 12.5% of all sequences, respectively. OTU 4 dominated during month −2 where it represented over 90% of all sequences. Among the other abundant OTUs, OTU 0, OTU 1 and OTU 2 predominated during months 0 and 4 but were almost absent at month 12. In contrast, OTU 3 was the most abundant at month 12 (Figure 5). This observation indicates that, despite the fact that cellulose was available during the entire decomposition process, specific cellulolytic fungi were present during different stages of decomposition. The abundance of one-half of the 30 most abundant OTUs significantly changed over time (Supplementary Table 2).

Phylogenetic analysis of the *cbhI* gene sequences showed that 38% of all sequences clustered with sequences of known fungal taxa with >70% bootstrap support (Supplementary Figure 1). Of these sequences, the majority belonged to the *Basidiomycota* (29%) phylum, which was represented by two OTUs, and the other 9% belonged to seven ascomycetous OTUs. Sequence similarities >97% allowed us to determine the taxonomic affiliation of OTU 11 to the ectomycorrhizal basidiomycete *Russula paludosa* and OTU 46 to the ascomycete *Aureobasidium pullulans*.

Discussion

During the decomposition of *Q. petraea* leaves used in this study, approximately 70% of the total mass was lost within 24 months (Figure 1; Šnajdr *et al.*, 2011). The C/N ratio decreased from 49 to 22 within 12 months and remained constant later. Fungal biomass increased rapidly from low values in the live and senescent leaves to a maximum at month 2 and remained lower but constant until the end of the experiment. The activity of cellulolytic enzymes was detected in live and senescent leaves, which indicates that decomposition started before leaf abscission (Figure 1; Šnajdr *et al.*, 2011). Three distinct decomposition phases have been distinguished that are characterised by the sequential mass loss of extractables and hemicelluloses, cellulose, and lignin (Supplementary Figure 2; Šnajdr *et al.*, 2011). This seems to be consistent with the culture-based observations that divided fungi into early, intermediate and late decomposers (Frankland, 1998; Osono and Takeda, 2001; Tang *et al.*, 2005; Osono, 2007). The culture-dependent studies, however, tend to underestimate the total diversity of fungi and are biased towards rapidly growing species (Hering, 1967; Frankland, 1998). Thus, they do not provide reliable information about fungal communities associated with leaves/litter during its degradation. Despite several limitations (see for example Amend *et al.*, 2010), next-generation sequencing seems to be better suitable to explore the fungal community because it can deliver information at higher quantitative resolution and is not biased towards easily culturable and fast growing taxa.

The live oak leaves used in this study harboured a relatively rich and even fungal community with its diversity comparable to previous reports from *Quercus macrocarpa* (Jumpponen and Jones, 2009a, b). The low biomass of the fungi on live leaves is possibly a consequence of the action of the

protective mechanisms of the plant. After leaf senescence, rapid proliferation of opportunistic *Mycosphaerella* spp. resulted in a sevenfold increase in fungal biomass but a rapid decrease in diversity. The rapid increase in fungal diversity after the litterfall was caused by the invasion of new colonisers and was detected during month 2 (Figure 1). Fungal diversity continued to increase until month 4, which indicates the arrival of new species on the substrate. However, the abundance of the most common fungal genera did not change significantly (Figure 2).

Fungi from the *Ascomycota* phylum prevailed in the live and senescent leaves on the trees (88.5% and 99.5% of amplicons, respectively). These data are in accordance with previous culture-based studies on various trees (Osono, 2002; Santamaría and Bayman, 2005) and the pyrosequencing analyses of live *Q. macrocarpa* leaves (Jumpponen and Jones, 2009a, b). However, the most common genera recorded from a *Q. macrocarpa* phyllosphere in North America were quite distinct on the genus level. Of the major genera, *Microsphaeropsis*, *Alternaria*, *Epicoccum, Aureobasidium*, *Phoma* and *Erysiphe* were detected, and only *Aureobasidium* and *Epicoccum* were recovered from live leaves in this study with a $>1\%$ frequency, which demonstrates that either the tree species, geographic distance or different environmental conditions affect the composition of phyllosphere mycoflora.

Some fungi associated with living tree leaves are also found in association with decomposing leaf litter (Koide *et al.*, 2005; Osono, 2006). The fact that some live leaf-associated fungi are able to produce extracellular enzymes or decompose sterile senescent leaves (Korkama-Rajala *et al.*, 2008; Žifčáková *et al.*, 2011) led to the hypothesis that certain taxa may change from endophytism to a saprotrophic strategy. In addition, molecular evidence indicates that fungi cultured from live leaves and decaying litter may indeed belong to the same taxa (Promputtha *et al.*, 2007). This study shows that phyllosphere fungi are still quantitatively important during the subsequent stages of decomposition, at least until month 4 (Figures 2 and 4). Establishment in live, nonsenescent leaves created an opportunity for these fungi to readily exploit leaf-derived nutrients during decomposition after leaf senescence. Fungi belonging to the genera *Holwaya*, *Cladosporium*, *Aureobasidium*, *Davidiella* and *Cryptococcus* were predominant in living oak leaves, in senescent leaves nearly disappeared and their abundance rose again in early phases of litter decomposition. The genera *Aureobasidium* and *Cladosporium* contain well-known phyllosphere fungi that have been repeatedly isolated from various trees (Sadaka and Ponge, 2003; Slavikova *et al.*, 2007; Unterseher and Schnittler, 2009), and their persistence until early decomposition has been reported (Sadaka and Ponge, 2003). OTUs belonging to the *Mycosphaerella* genus, which comprises pathogenic and saprotrophic species (Suto, 1999), were the most dominant in live leaves and made up a significant portion of the population at month 0 where they represented 90% of sequences, which indicates that they are both endophytes and efficient early saprotrophs.

The first year of our experiment was characterised by a relatively rapid loss of litter mass, a decrease in the C/N ratio and the cellulose content, and a relatively high activity of cellulolytic enzymes, which causes faster decomposition of cellulose (Supplementary Figure 2). These conditions were associated with the continuous dominance of fungi from the *Ascomycota* phylum, which are generally known to selectively decompose cellulose over lignin. Dominance of ascomycetous fungi in the early stages of beech litter decomposition was currently also demonstrated using metaproteomic approach (Schneider *et al.*, 2012). Similar results were obtained when 8-week-old *Fagus sylvatica* litter was analysed, except that it also contained a significant proportion of fungi from the *Mucoromycotina* phylum (Aneja *et al.*, 2006). Despite the fact that the *Mucoromycotina* fungi are often considered to be opportunistic microorganisms that are associated with nutrient-rich substrates, their abundance in *Q. petraea* litter was low until month 8. Among the fungal genera that were dominant during month 2, *Naevala*, *Cryptococcus* and *Mycosphaerella* were detectable in the live leaves, and the basidiomycetous genera *Athelia* and *Mrakia* appeared anew during month 2 and immediately became dominant, which demonstrates their ability to rapidly proliferate on fresh litter. *Naevala*, *Athelia* and *Cryptococcus* fungi maintained their prevalence until month 4, whereas *Mrakia* fungi nearly disappeared and were replaced by *Polyscytalum* and *Rhodotorula* fungi. Month 8 was characterised by an entirely different fungal community, which was most likely caused by a depletion of the majority of the readily available organic compounds and was associated with a sharp decrease in the phyllosphere fungi. Fungi belonging to the *Glomeromycota* and the *Mucoromycotina* phyla were detectable on litter beginning at month 8, and their abundances gradually increased until the end of the experiment. The fungal genera *Troposporella*, *Guignardia*, *Ramariopsis*, *Sympodiella* and *Cryptococcus* prevailed at month 8. *Troposporella* fungi have been recorded in seasonally flooded soil ecosystems (Carrino-Kyker and Swanson, 2008), where they were most likely involved in the decomposition of allochthonous carbon input. *Troposporella* remained frequent until month 12, whereas basidiomycetous *Sistotrema* and *Rhodocollybia* and lichenised ascomycetous *Dimelaena* fungi appeared for the first time at this stage. *Rhodocollybia* is a typical saprotroph (Valášková *et al.*, 2007), and the polyphyletic genus *Sistotrema* contains both ectomycorrhizal and decomposer fungal species (Di Marino *et al.*, 2008; Boberg *et al.*, 2011).

During the second year, the rate of litter mass loss was relatively slow and the activity of cellulolytic enzymes decreased, which indicated that the easily accessible polysaccharides were depleted. Also, the substrate was richer in the recalcitrant lignin and nitrogen and characteristic with the increased activity of ligninolytic enzymes (Supplementary Figure 2). Fungi from the *Basidiomycota* phylum distinctively dominated over fungi from the *Ascomycota* phylum at month 24. In previous studies, basidiomycetous species, particularly the saprotrophic cord formers, have often been demonstrated to be late litter decomposers (Osono, 2007; Duong *et al.*, 2008) because of their capability to synthesise enzymes required for the degradation of complex polymers (Baldrian, 2008). Interestingly, basidiomycetous cord formers were not among the most frequent taxa observed in our study. Instead, the basidiomycetous yeast genus *Trichosporon* comprised 50% of all sequences at month 24. This is consistent with a recent report that identified this species as the second most abundant cellulose decomposer in litter using stable isotope probing (Štursová *et al.*, 2012). Although the cellulose content of the litter at this stage is relatively low (Šnajdr *et al.*, 2011), it may be still sufficient to support the growth of yeasts and may not be sufficient to support the growth of the large mycelia of basidiomycetous cord formers. There is only a small overlap of the fungal community in litter and in the uppermost (organic) soil horizon at the site of study (unpublished data) with >50% of fungi in soil being ectomycorrhizal. Among the genera reported in this study, *Mortierella*, *Cryptococcus*, *Trichosporon*, *Ambispora* and *Naevala* are also frequent in soil, which can serve as a reservoir for their spread.

This study demonstrated that fungal succession during litter decomposition is much faster than so far expected from the culture-based studies (Figure 2). The fast appearance–disappearance of fungal taxa seems to contrast with the reported persistence of DNA from inactive fungi in decaying wood (Rajala *et al.*, 2011) and to support the rapid turnover of early/intermediate/late saprotrophs (Lindahl and Finlay, 2006). The successional changes are likely governed not only by the relatively slow changes of the polysaccharide, lignin and nitrogen content in litter but possibly by other factors including more subtle changes in litter chemistry and interspecific fungal interactions.

Cellulose is the major polysaccharide in plant litter, and cellulose-degrading enzymes are thus an obvious target for the study of the decomposing microorganisms. Among these, cellobiohydrolases (exocellulases), which catalyses the rate-limiting step of cellulose decomposition (Baldrian and Valášková, 2008), and the *cbhI* gene represent suitable markers for the study of cellulolytic fungi (Edwards *et al.*, 2008; Weber *et al.*, 2011; Baldrian *et al.*, 2012). Here, we show that several exocellulase-producing fungi are present in the live leaves of *Q. petraea* although the community is dominated by a single species (Figure 5). This OTU of *cbhI* genes was assigned to fungi from the *Basidiomycota* phylum, which is surprising if we consider the dominance of fungi from the *Ascomycota* phylum at this stage. The diversity of cellulolytic fungi is high in senescent leaves with 58 observed and >200 predicted OTUs. As the leaves are still attached to the trees, these fungi must have colonised the substrate before its contact with soil. Later in decomposition, estimates of *cbhI* richness were approximately 200 in number, which indicates that there are approximately 100 cellulolytic fungal species when multiple copies of the gene per fungal genome are considered (Edwards *et al.*, 2008; Weber *et al.*, 2011). The overall fungal diversity did not correlate with the diversity of fungi harbouring the *cbhI* gene, which indicates that the proportion of cellulolytic fungi changes during decomposition; no clear link was observed between the diversity of the *cbhI* genes and the activity of cellobiohydrolase. Although cellulose represents a substrate that is present in the litter during the entire decomposition process, the community of cellulolytic fungi also showed successive changes similar to those of the total fungal community with dominant OTUs appearing and disappearing (Figure 5). This may indicate that the individual cellulolytic fungi have specific additional nutritional requirements or competitive abilities. Interestingly, the sequences dominating in the *cbhI* gene pool at month 12 belonged to the ectomycorrhizal genus *Russula*. Our results are consistent with previous observations that identified cellulases and class II peroxidases in these fungi (Bodeker *et al.*, 2009; Štursová *et al.*, 2012) that may combine the mycorrhizal and saprotrophic lifestyle to some extent.

This study demonstrates that the composition of the fungal community changes with changing litter quality much faster than previously thought. Furthermore, similar changes during the decomposition process are observed among cellulolytic fungi, which indicates that succession is not only driven by the availability of the major nutrient sources but also by other factors, perhaps other nutritional requirements or the competitive abilities of individual taxa. The initial steps of decomposition where fungi dominate the decomposer community are characterised by a high involvement of fungi that occur on the live leaves of the tree. However, further research on both the structural and functional aspects of fungal community composition, for example, use of the metatranscriptomic or metaproteomic approaches, are needed to better understand the functional role of individual fungal taxa during decomposition.

Conflict of Interest

The authors declare no conflict of interest.

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Seasonal dynamics of fungal communities in a temperate oak forest soil

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Summary

• Fungi are the agents primarily responsible for the transformation of plant-derived carbon in terrestrial ecosystems. However, little is known of their responses to the seasonal changes in resource availability in deciduous forests, including photosynthate allocation belowground and seasonal inputs of fresh litter.
• Vertical stratification of and seasonal changes in fungal abundance, activity and community composition were investigated in the litter, organic and upper mineral soils of a temperate *Quercus petraea* forest using ergosterol and extracellular enzyme assays and amplicon 454-pyrosequencing of the rDNA-ITS region.
• Fungal activity, biomass and diversity decreased substantially with soil depth. The highest enzyme activities were detected in winter, especially in litter, where these activities were followed by a peak in fungal biomass during spring. The litter community exhibited more profound seasonal changes than did the community in the deeper horizons. In the litter, saprotrophic genera reached their seasonal maxima in autumn, but summer typically saw the highest abundance of ectomycorrhizal taxa. While the composition of the litter community changes over the course of the year, the mineral soil shows changes in biomass.
• The fungal community is affected by season. Litter decomposition and phytosynthate allocation represent important factors contributing to the observed variations.

Introduction

Temperate forests are one of the major biomes on earth, covering an area of 570 million ha and thus playing an important role in the global C budget (FAO & JRC, 2012). In forest ecosystems, carbon enters the soil in the form of plant litter (Berg & McClaugherty, 2003), through the belowground allocation of C fixed by plant photosynthesis (Högberg *et al.*, 2010) and as a dead fungal and animal material. Fungi play the primary role in regulating the flow of C through all of these pathways. Saprotrophic fungi decompose organic matter due to their ability to produce a wide range of extracellular enzymes (Steffen *et al.*, 2007; Baldrian *et al.*, 2011), which allows them to efficiently attack the recalcitrant lignocellulose matrix that other organisms are unable to decompose (Boer *et al.*, 2005). Mycorrhizal fungi, as obligate symbionts, acquire access to the C

compounds derived from the photosynthates of their host plants (Hobbie, 2006) in exchange for soil-derived nutrients (van der Heijden & Horton, 2009), and they also contribute directly to the carbon enrichment of soils by mediating the belowground allocation of C from plant roots to soil (Clemmensen *et al.*, 2013).

In temperate deciduous forests, as a consequence of the input of new litter and its transformation, it is possible to recognise three distinct compartments in the soil profile: (i) the litter (L horizon), containing organic matter derived from dead plant biomass almost exclusively; (ii) the organic (or humic) H horizon, representing a mixture or processed plant-derived organic matter and soil components; and (iii) the mineral soil horizon, with a lower content of organic matter originating both from the decomposition of organic matter and exudation from the abundant tree roots. If invertebrate mixing is limited, the age of the litter-derived organic material increases with soil depth as decomposition progresses, and this is accompanied by changes in its chemical composition, leading to increasing recalcitrance and the formation of humic compounds (Šnajdr *et al.*, 2008). The vertical distribution of the fungal community in boreal and temperate forests has been demonstrated to reflect soil stratification: saprotrophic taxa are more abundant close to the surface of the forest floor where most carbon is mineralised, while mycorrhizal fungi increase in abundance with soil depth, where they mobilise nitrogen to be supplied to the roots of plants (O'Brien *et al.*, 2005; Lindahl *et al.*, 2007).

Observations from diverse forest soils suggest that environmental factors such as temperature, water availability and substrate quality may be important factors affecting microbial community composition (Aponte *et al.*, 2010; Kaiser *et al.*, 2010; Landesman & Dighton, 2011; Kuffner *et al.*, 2012). Temperate deciduous forests are characterised by the photosynthetic activity of trees during the vegetative period and a short period of litterfall in autumn, when fresh litter with easily available nutrients accumulates on the forest floor (Šnajdr *et al.*, 2011). These seasonal processes then underlie the seasonality of soil carbon allocation and its availability to the soil biota (Högberg *et al.*, 2010; Kaiser *et al.*, 2010). Belowground carbon allocation via plant roots exhibits several-fold seasonal differences with a maximum during the late vegetative season (Högberg *et al.*, 2010).

Seasonal variations of fungal communities have been widely studied, however the methods used were unable to sufficiently characterize fugal community structure or authors focused only on particular soil horizon or group of fungi. Prior studies were mainly based on traditional approaches such as enzyme assays or assessment of microbial biomass (Šnajdr *et al.*, 2008; Baldrian *et al.*, 2013a; Berg *et al.,* 1998; Björk *et al.,* 2008). There have thus far been several reports concerning specific functional groups of fungi (Koide *et al.,* 2007; Rosling *et al.* 2003; Courty *et al.*, 2008; Courty *et al.*, 2010) or studies limited to particular soil or litter horizons (Jumpponen *et al.*, 2009; Dumbrell *et al.*, 2011; Davey *et al.*, 2012; Coince *et al.*, 2013). However the thorough knowledge of seasonal influences on fungal communities in temperate deciduous forest soil with respect to vertical stratification is missing. Understading of seasonal dynamic of fungal community and its functioning in forest soil ecosystem is necessary for prediction its response to global changes, considering soils as a sink of carbon dioxide. Moreover even if detailed descriptions of fungal communities by soil horizons exist for some ecosystems (Baldrian *et al.*, 2012; Uroz *et al.*, 2013), it is unclear how representative these studies might be based on a single sampling.

In this study, an analysis of fungal community composition, abundance and the activity of extracellular enzymes was performed for the upper horizons of a deciduous *Quercus petraea* forest. The primary goal of this study was to describe

seasonal variations in the fungal community composition in the context of changing resource availability throughout the seasons. To achieve this, sampling was performed in spring (early May), shortly after leaf appearance; in summer (July), along with the highest temperatures and high photosynthetic production; in autumn (October), in the middle of the litterfall period; and in winter (February; Fig. 1). In addition to changes in tree productivity, the amount and quality of litter changed from the input of freshly fallen leaves (approx. 4 t ha^{-1}, unpublished results), whose composition supports fast decomposition (October), to a litter horizon depleted of easily decomposable compounds (July). We hypothesised that the structure of the fungal community would reflect the availability of nutrients in the soil profile horizons. Based on a previous study, where considerable temporal shifts in fungal community structure during the decomposition of oak litter were observed, we anticipated similar changes in the litter horizon because the last year's litter represents a considerable percentage of the total litter mass (Voříšková & Baldrian, 2013). In the deeper horizons, we expected a shift from a high relative abundance of ectomycorrhizal taxa during the vegetative season to a high proportion of saprotrophic taxa in the absence of root C allocation because our previous study showed that saprotrophic taxa are more metabolically active during the period when photosynthesis does not occur (Baldrian *et al.*, 2012). We also intended to answer questions concerning the suitability of the one-time surveys that are frequently reported for the description of fungal community composition within an ecosystem.

Materials and methods

Study site and sample collection

The study site was an oak (*Q. petraea*) forest in the Xaverovský Háj Natural Reserve, near Prague, Czech Republic (50°5'38" N, 14°36'48" E). The site was previously studied with respect to decomposition-related extracellular enzymes in the forest topsoil (Šnajdr *et al.*, 2008; Baldrian *et al.*, 2010; Baldrian *et al.*, 2013a), as well as the decomposition of litter and associated changes in fungal community composition (Voříšková & Baldrian, 2013). The soil was an acidic cambisol with developed L, H, Ah and A horizons. Sampling of the topsoil was performed in the spring (9 May, approximately two weeks after the emergence of leaves), summer (29 July), autumn (28 October, during the late phase of litterfall) and winter (19 February; Fig. 1). Soil samples were collected in four defined plots (10 m^2, approximately 100 m from each other) of the sampling site. Six soil cores (4.5 cm in diameter) were collected at each sampling plot and were divided into L horizon (~ 0.5-1 cm, 3.5), H horizon (~ 1-3 cm) and Ah - soil horizon (upper portion, up to a depth of 5 cm). Samples of the L horizon were cut into approx. 0.25 cm2 pieces, while the soil samples were sieved using a 2-mm sieve. The resulting material was combined to yield a composite sample from each horizon and plot. Subsamples for chemical analyses, quantification of microbial biomass and DNA extraction were frozen and stored at -45 °C, subsamples for enzyme analysis were stored at 4°C.

Sample analysis

Enzyme assays were performed within 48 h on samples extracted using 160 mM phosphate buffer, pH 7 and desalted using Sephadex columns, as previously described (Šnajdr *et al.*, 2008). Briefly, laccase was assayed using the oxidation of 2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, manganese peroxidase using 3-methyl-2-benzothiazolinone hydrazone and 3,3-dimethylaminobenzoic acid in the presence of Mn and hydrogen peroxide, endocellulase, and endoxylanase (EC 3.2.1.8) were measured using azo-dyed carboxymethyl cellulose and birchwood xylan and the activities of all other enzymes

with *p*-nitrophenyl-based substrates. All enzyme assays were performed at pH 5 except for laccase where the pH of the buffer was 4.5. One unit of enzyme activity was defined as the amount of enzyme forming 1 mmol of reaction product per min.

Dry mass content was measured after drying at 85 °C, organic matter content after burning at 650 °C, and pH was measured in distilled water (1:10). C and N contents were measured using an elemental analyser.

Total ergosterol was extracted with 10% KOH in methanol and analysed by HPLC (Šnajdr et al 2008) using a method modified from (Bååth, 2001).

454-Pyrosequencing of fungal internal transcribed spacer (ITS)

Total genomic DNA was extracted from 300 mg of soil material using a modified method according to (Sagova-Mareckova *et al.*, 2008). The primers ITS1/ITS4 (White *et al.*, 1990) were used to amplify the ITS1 region, the 5.8S ribosomal DNA and the ITS2 region of the fungal ribosomal DNA. The primer pair used in this study is especially suitable for the analysis of *Ascomycota*, *Basidiomycota*, *Mucoromycotina* and *Mortierellomycotina* while it may be biased against some members of the *Glomeromycota* and the *Chytridiomycota*. A two-step PCR amplification using composite primers containing multiplex identifiers (Baldrian *et al.*, 2012) was performed to obtain amplicon libraries for 454-pyrosequencing. In the first step, each of three independent 25 µl reactions per DNA sample contained 2.5 µl of 10x polymerase buffer, 1 µl of each primer (0.01 mM), 0.5 µl of PCR Nucleotide Mix (10 mM) and 0.25 µl of polymerase (2 U μl^{-1}; Pfu DNA polymerase:OmniTaq DNA polymerase, 1:24). The cycling conditions were 94°C for 5 min; 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 70 °C for 1 min; followed by 70 °C for 10 min. Pooled PCR products were purified using a MinElute PCR Purification Kit (Quiagen, Hilden, Germany). The product of the first PCR was used as a template for the second PCR. In the second step, one 50 µl reaction per DNA sample contained 5 µl of 10× polymerase buffer, 1.5 µl of DMSO for PCR, 0.4 µl of forward fusion primer (ITS1, tag sequence, 454-specific sequence), 0.4 µl of reverse fusion primer (ITS4, 454-specific sequence), 1 µl of PCR Nucleotide Mix, 1.5 µl of polymerase (2 U μl^{-1}; Pfu DNA polymerase:Dynazyme DNA polymerase, 1:24) and 100 ng of template DNA. The cycling conditions were 94°C for 5 min; 10 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min; followed by 70 °C for 10 min. PCR products were purified using Agencourt AMPure XP (Beckman Coulter, Beverly, MA). The concentration of PCR products was quantified using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), and an equimolar mix of PCR products from all samples was prepared. The mixture of PCR products was separated by electrophoresis and gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), followed by purification using Agencourt AMPure XP and a MinElute PCR Purification Kit to remove primer-dimers. The amplicons were subjected to sequencing on a GS Junior 454-pyrosequencer (Roche, Basel, Switzerland).

Bioinformatic analysis

The pyrosequencing data were processed using the pipeline SEED with respect to the proposed procedures of standardized data analysis (Nilsson *et al.*, 2011; Větrovský & Baldrian, 2013). Pyrosequencing noise reduction was performed using the Denoiser 0.851 (Reeder & Knight, 2010), and chimeric sequences were detected using UCHIME (Edgar *et al.*, 2011) and deleted. The sequences were shortened to 380 bases and clustered using Usearch (Edgar, 2010) at a 97% similarity level. Consensus sequences were constructed for each cluster, and the OTUs were constructed by clustering these consensus sequences at

97% identity (Lundberg *et al.*, 2012). The abundance data reported in this paper are based on this dataset of sequence abundances and should be taken as proxies of taxon abundances only with caution (Lindahl *et al.,* 2013). Closest hits were identified using the PlutoF pipeline (Tedersoo *et al.*, 2010); non-fungal sequences (< 1% were disregarded). Sequence data have been deposited in the MGRAST public database (http://metagenomics.anl.gov/, data set number 4524551.3).

Diversity and statistical analysis

The Shannon-Wiener Index and the amount of the most abundant OTUs that represented 80% of all sequences were used as diversity estimates, providing combined information on species richness and evenness at particular sampling depths. These estimates were calculated for a data set containing 1,800 randomly chosen sequences from each sample. Since the fungal communities at individual plots differed and because these among-plot differences might have hidden the differences among seasons, comparisons of seasonal abundances were performed after normalisation of the abundances of each fungal taxon using the mean abundance in the particular plot and horizon during all seasons. Because the majority of taxa were represented by a very small number of reads and because such read counts were demonstrated to not be technically reproducible (Lundberg *et al.*, 2012), only taxa with higher relative abundances $\geq$ 0.5% in $\geq$ 5 samples were tested for seasonal variations in abundance. The plot-normalised abundances of these measurable taxa were also subjected to a PCA along with environmental variables. The Jaccard Index (JI) calculated for all OTUs with relative abundances $\geq$ 0.5% in at least one sample was used as a measure of community similarity (Koleff *et al.*, 2003). The Jaccard Index is calculated as A/(A+B+C), where A is the number of species found in both of the samples, and B and C represent the number of species unique to either of the two samples analysed. The JI ranges from 0 (no species shared) to 1 (all species shared). The pipeline SEED (see above) was used for data pre-processing and diversity calculations and Statistica 7 (Statsoft, USA) was used for statistical analyses. A one-way analysis of variance with the Fisher's least significant difference *post hoc* test was used to analyse the statistical significance of differences among groups of samples. Differences with a $P < 0.05$ were regarded as statistically significant.

Results

Soil properties, activity of extracellular enzymes and fungal biomass

The soil properties changed substantially with soil depth: the organic matter content decreased from 82% in the L to 42% and 16% in the H and Ah horizons, respectively, and N content showed similar inter-horizon trend. With depth, the soil dry mass content increased and the soil pH decreased. With decreasing organic matter content in the soil, the activity of extracellular enzymes also decreased, being 4-40× lower in the Ah horizon than in the L horizon. Fungal biomass in the H horizon was 6×, and in the Ah, it was 23× lower than in the litter; the fungal biomass content per g organic matter decreased with depth from 188 μg g^{-1} to 76 μg g^{-1} and 50 μg g^{-1} (Fig. 2).

Fungal biomass was similar among the seasons in the H horizon, ranging 26-36 μg g^{-1} soil dry mass. In the L horizon, the ergosterol content was highest in spring (272 μg g^{-1} compared to the minimum of 128 μg g^{-1} in the autumn). In the Ah horizon, the ergosterol content was significantly increased in summer (14 μg g^{-1}), while it was only between 5-6 μg g^{-1} during the winter and spring (Fig. 1). The majority of the enzymes studied showed their highest activity in winter, especially in the L and H horizons. The exception was

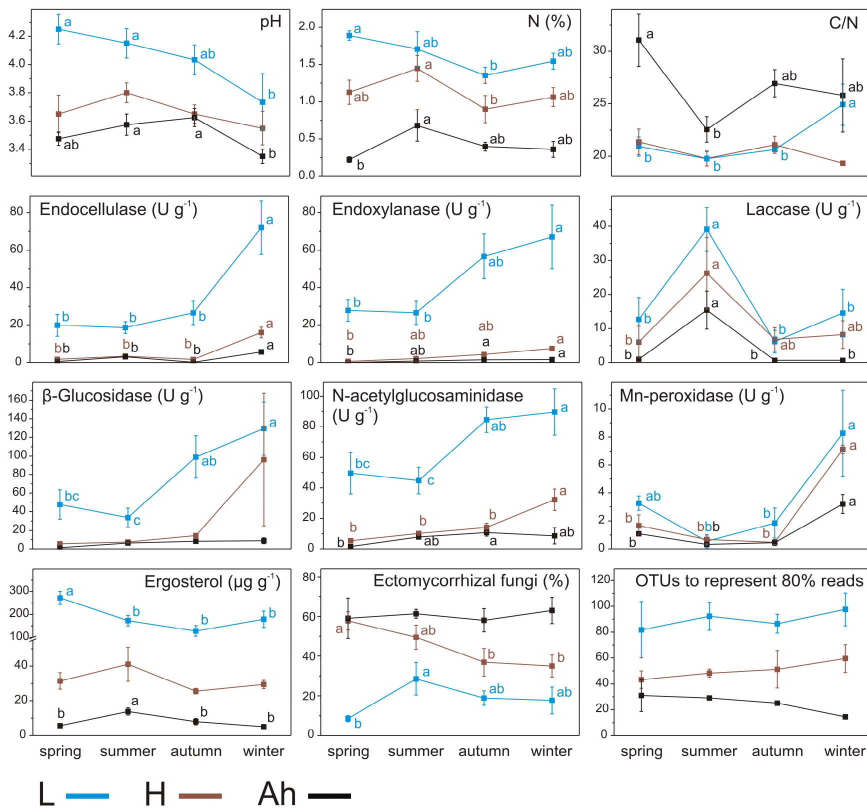


Fig. 1.: Seasonal properties of *Quercus petraea* forest soil by season in the L, H and Ah horizons. Seasonal trends of ectomycorrhizal community are based on their relative abundance in the entire community. Data represent means of four replicates with standard errors. Statistically significant differences among seasons are indicated by different letters.

laccase, whose activity was highest in summer (Fig. 1).

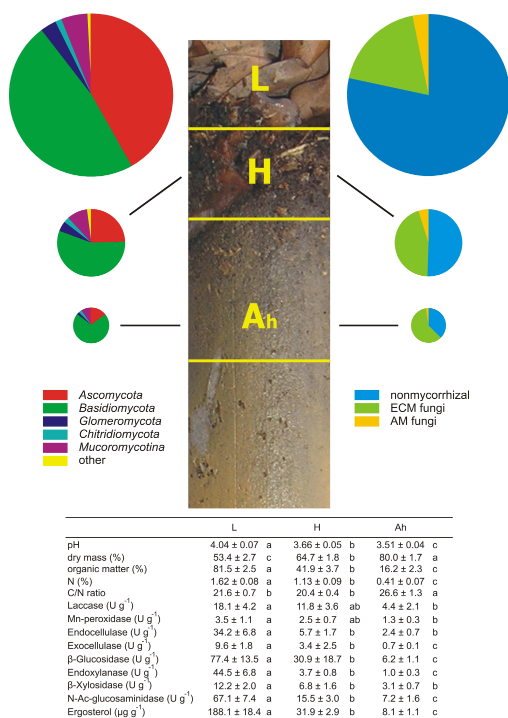
Fungal community composition

In total, 213,339 raw sequences were obtained from 454-pyrosequencing, of which 135,830 remained for analysis after quality-filtering, de-noising and the removal of short and chimeric sequences and sequences not belonging to fungi (the latter accounted for < 1% of the total). An average of 2,830 sequences were obtained (minimum 1,803) per sample. All of the sequences clustered into 8,264 OTUs (including 5,730 singletons) at a 97% similarity threshold (Supporting Information Table S1). Fungal diversity, expressed as the Shannon-Wiener index calculated at 1,800 sequences / sample, decreased from the L (4.51 ± 0.49) to the H horizon (4.05 ± 0.36, P = 0.003) and from the H to the Ah horizon (3.43 ± 0.40, P < 0.0001). Seasonal differences were not observed except in the Ah horizon, where summer communities were marginally more diverse than winter communities (P = 0.06). Community evenness, expressed as the number of the most abundant OTUs that represented 80% of all of the sequences in each sample, also decreased significantly with soil depth and did not show seasonal variations (Fig. 1).

The fungal communities at each plot and season were more similar between the H and Ah horizons (mean Jaccard Index 0.513) than between the L and H horizons (mean JI 0.451; P = 0.013 that the similarity expressed as the Jaccard Index between L/H and H/Ah samples from the same season and plot is the same). The L horizon communities were more similar among plots for each season than were the samples from the two deeper horizons (mean JI for L 0.488, H 0.402, and Ah 0.379; P < 0.001 that the JI for each season across plots in L is the same as in H and Ah), and they were also more similar for each plot across seasons (JI 0.466, 0.422, and 0.397, P < 0.017 that the for each plot across seasons in L is the same as in H and Ah).

The overall fungal community was dominated by sequences assigned to the *Basidiomycota* (58%) and *Ascomycota* (27%). The *Mucoromycotina* were represented in 7.9% of all sequences, and fungi from the *Glomeromycota* and *Chitridiomycota* comprised 3.4% and 2.2% of all sequences, respectively. Sequences from the *Ascomycota* and *Basidiomycota* demonstrated comparable counts in the L horizon – 42% and 48%. With soil depth, the abundances of the ascomycetous sequences decreased and those of the basidiomycetous fungi increased to reach 15% and 71%, respectively, in the Ah horizon (Fig. 2). The most abundant fungal orders were the basidiomycetous *Russulales* (25%), *Agaricales* (11%) and *Tremellales* (8.7%). Members of the orders *Agaricales*, *Helotiales* and *Tremellales* dominated in samples from the L horizon. The abundances of the *Agaricales* and *Tremellales* did not change significantly with increasing soil depth, while the abundance of the *Helotiales* decreased. In contrast, the H and Ah horizons contained more sequences belonging to the ectomycorrhizal *Russulales*, their relative abundance in the Ah being 6 times higher than in the L horizon.

In total, 757 fungal genera were identified as being the best hits for the OTUs for the whole dataset. The most abundant fungal genera in the litter horizon were the saprotrophic *Mycena*, *Sistotrema* and *Cryptoccocus*, while the deeper horizons were enriched with fungi belonging to the ectomycorrhizal genera *Russula* and *Lactarius*. The genus *Russula* represented 18% of all the sequences in the H horizon and 31% in the Ah horizon; the most abundant OTUs across all samples also belonged to *Russula* (OTU133, 100% similarity to *R. atropurpurea*) and *Lactarius* (OTU002, 98% similarity to *L. quietus*; Supporting Information Table S1). Fungi in the litter were apparently more influenced by seasonal effects than were those in the deeper horizons: 59% of the abundant fungal genera (among them, 8



	L		H		Ah	
pH	4.04 ± 0.07	a	3.66 ± 0.05	b	3.51 ± 0.04	c
dry mass (%)	53.4 ± 2.7	c	64.7 ± 1.8	b	80.0 ± 1.7	a
organic matter (%)	81.5 ± 2.5	a	41.9 ± 3.7	b	16.2 ± 2.3	c
N (%)	1.62 ± 0.08	a	1.13 ± 0.09	b	0.41 ± 0.07	c
C/N ratio	21.6 ± 0.7	b	20.4 ± 0.4	b	26.6 ± 1.3	a
Laccase (U g^{-1})	18.1 ± 4.2	a	11.8 ± 3.6	ab	4.4 ± 2.1	b
Mn-peroxidase (U g^{-1})	3.5 ± 1.1	a	2.5 ± 0.7	ab	1.3 ± 0.3	b
Endocellulase (U g^{-1})	34.2 ± 6.8	a	5.7 ± 1.7	b	2.4 ± 0.7	b
Exocellulase (U g^{-1})	9.6 ± 1.8	a	3.4 ± 2.5	b	0.7 ± 0.1	c
β-Glucosidase (U g^{-1})	77.4 ± 13.5	a	30.9 ± 18.7	b	6.2 ± 1.1	c
Endoxylanase (U g^{-1})	44.5 ± 6.8	a	3.7 ± 0.8	b	1.0 ± 0.3	c
β-Xylosidase (U g^{-1})	12.2 ± 2.0	a	6.8 ± 1.6	b	3.1 ± 0.7	b
N-Ac-glucosaminidase (U g^{-1})	67.1 ± 7.4	a	15.5 ± 3.0	b	7.2 ± 1.6	c
Ergosterol (µg g^{-1})	188.1 ± 18.4	a	31.9 ± 2.9	b	8.1 ± 1.1	c

Fig. 2.: Characterisation of the L, H and Ah horizons of *Quercus petraea* forest soil. Mean abundances of higher fungal taxa and fungal life strategies (the area of the charts corresponds to the ergosterol content). Data on soil chemistry, activity of extracellular enzymes and ergosterol content are means of 16 replicates with standard errors. Statistically significant differences among horizons are indicated by different letters.

of the top 10) showed statistically significant differences in their seasonal abundance, compared with 29% and 32% in the H and Ah horizons, respectively (Supporting Information Table S2).

The seasonal differences among the relative abundances of fungal genera in the L horizon were profound: the saprotrophic genus *Mycena* was represented by only 0.5% of the sequences in winter, while it represented 16% in spring, *Mycosphaerella* represented 0.04% in summer but 8% in autumn, and *Naevala* represented 0.01% in summer and 3.5% in winter. For most ectomycorrhizal fungi, low relative abundances were recorded in spring (*Russula* 1%, *Lactarius* 0.3%, *Amanita* 0.05%), while high abundances were recorded in summer (*Russula* 7%, *Lactarius* 6%, *Amanita* 3.4%; Supporting Information Table S2). These seasonal changes were also demonstrated in the PCA analysis where samples from winter clustered separately from the other seasons (Fig. 3). In the autumn, when fresh litter accumulated on the forest floor, the saprotrophic genera *Mycosphaerella*, *Mucor*, *Geomyces*, *Umbelopsis* and *Lachnellula* reached their seasonal maxima. The highest activity of most enzymes was recorded in winter, as well as the highest C/N ratio. This finding was accompanied by the highest abundances of the saprotrophic genera *Cryptococcus*, *Rhodotorula*, *Naevala*, *Fulvoflamma* and *Kriegeria*. The other saprotrophic genera *Mycena*, *Cladophialophora* and *Meliniomyces* were abundant during spring, the season with the highest fungal biomass in the litter. Finally, summer (the driest season, with high laccase activity) typically demonstrated the highest abundances for all ECM taxa: *Russula*, *Lactarius*, *Tomentella*, *Amanita* and *Hygrocybe,* except for *Xerocomus,* which was highest in autumn. Not surprisingly, the proportion of sequences for the ectomycorrhizal fungi was highest in summer at 28.6%, which was significantly higher than in spring (8.5%).

In the H horizon, PCA showed a clear separation between winter and spring samples along the first axis. Winter was characterised by high activities of most extracellular enzymes and by the lowest proportion of ECM fungi (35%), while spring exhibited the highest proportion of ECM sequences (58%). In the Ah horizon, PCA separated summer samples from those of spring and winter. The proportion of ECM did not show significant fluctuations, but the proportions of individual ECM fungi varied seasonally. The highest activities of several enzymes, but the lowest fungal biomass, were observed in winter (Supporting Information Fig. S1).

Discussion

Forest soils represent an environment that exhibits distinct and sharp vertical stratification. The ultimate cause is likely to be the decrease in organic matter with soil depth due to the accumulation of litter on the soil surface and its gradual decomposition together with temperature and moisture content differences. The decrease in soil organic matter content is accompanied by a decrease in microbial biomass and in the rates of microbial processes such as respiration and the activities of extracellular enzymes (Agnelli *et al.*, 2004; Šnajdr *et al.*, 2008; Baldrian *et al.*, 2013a). In this study, soil organic matter decreased by a factor of five between the L and Ah horizons. The fact that fungal biomass in the Ah horizon was 23× lower than in the L likely reflects changes in the quality of the organic matter. Taking into account the ergosterol/fungal biomass ratio of 3.8 mg g^{-1} fungal biomass (Baldrian *et al.*, 2013b), fungal biomass might represent as much as 6.1% of the organic matter in the litter horizon; in the H and Ah horizons, this biomass would be less, namely 2% and 1.2%, respectively. Previous studies have also shown that the C/N ratio decreases with soil depth in certain soils (Baldrian & Štursová, 2011; Yang & Luo, 2011). During the process of decomposition, carbon from freshly fallen litter is released as CO_2, and if the N is retained, its relative proportion increases. For example, fresh *Quercus petraea* litter has a C/N ratio of 25, compared with 13-17 after *in vitro* degradation by saprotrophic fungi (Steffen *et al.*, 2007). Here, we

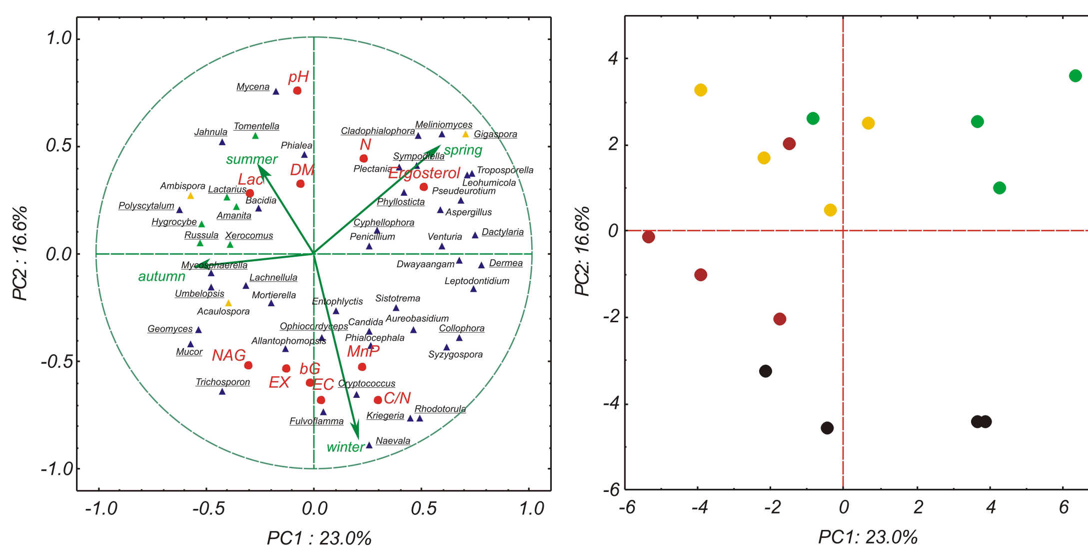


Fig. 3.: Principal component analysis of the plot-normalised relative abundances of fungal genera in the L horizon, seasonal loads and environmental variables. All genera with > 0.5% abundance in > 4 samples were considered. Only environmental variables showing significant differences among seasons were considered; fungal genera with significant seasonal variations in abundance are underlined. Ectomycorrhizal fungi are indicated in green, arbuscular mycorrhizal fungi in yellow. bG – β-glucosidase, DM – dry mass content, EC – endocellulase, EX – endoxylanase, Lac – laccase, MnP – Mn peroxidase, N – nitrogen, NAG – N-acetylglucosaminidase. Right panel shows the PCA loads of samples from individual seasons: spring – green; summer – yellow; autumn – brown; winter – black.

observed the highest C/N ratio in the Ah horizon, a fact that might support the importance of the allocation of C from tree roots into deeper soil as proposed by Clemmensen *et al.* (2013), who demonstrated that 70% of soil carbon was root-derived and was thus allocated from plants into the soil by mycorrhizal fungi.

Alternatively, the increased C/N ratio in mineral soil might be due to the depletion of N in the bulk soil as a result of its allocation to plants by ECM fungi.

The fungal community structure differed substantially among the three horizons studied. Litter-associated communities exhibited higher similarity among sampling plots than did soil communities. Within each plot, the communities of the deeper H and Ah horizons were more similar to each other than to the L horizon community, mainly due to the comparable abundances of ectomycorrhizal taxa. Interestingly, our study showed a substantial, significant decrease in community diversity: the amount of OTUs representing 80% of the fungal community was 90 in the L horizon, 51 in the H horizon and 25 in the Ah horizon (Fig. 1). Similar reductions in fungal diversity had previously been demonstrated in prairie soils, but a reduction in the Shannon index was only observed over several tens of cm of soil depth (Jumpponen *et al.*, 2010). In the forest ecosystems, the higher chemical heterogeneity of nutrient sources in the litter horizon (composed of material of various ages) might be the prerequisite for high diversity; the cause of higher diversity in the H horizon than in the Ah horizon might be the co-presence of organic substrates and mycorrhizal tree roots in the former. However, previous papers reporting on other forest ecosystems did not show differences in fungal diversity among horizons (O'Brien *et al.*, 2005; Baldrian *et al.*, 2012).

With respect to the abundance of the major functional groups of fungi, our results are in accordance with previous studies from boreal and temperate forest soils in that the relative proportion of ectomycorrhizal taxa (and thus, the *Basidiomycota*) increases with soil depth (Lindahl *et al.*, 2007; Edwards & Zak, 2010; Baldrian *et al.*, 2012; Clemmensen *et al.*, 2013). However, interestingly, if we consider the absolute amounts of fungal biomass, the highest ECM biomass per g soil dry mass, despite its lower proportion, was present in the L horizon. The supply of C from trees to the ECM fungi thus contributes substantially to the formation of fungal biomass in the litter. The fact that the ECM biomass in litter is produced from root-supplied C and is not due to saprotrophic litter transformation is supported by the substantial increase in the proportion of ECM in the litter from spring to summer. Summer would then also be the season with the highest total ECM biomass when considering the whole soil profile.

Despite the increasing evidence that enzyme activity shows seasonal variation (Wittmann *et al.*, 2004; Baldrian *et al.*, 2013a), as does the C allocation belowground (Ekblad *et al.*, 2013), the influence of these factors on soil fungal communities has never been addressed in sufficient detail. Several studies focused on seasonal variations of ECM fungi (Buée *et al.,* 2005; Courty *et al.,* 2008; Koide et al 2007), moreover Parrent and Vilgalys (2007) showed their seasonality across several years. However little information is thus far available about the seasonality of entire fungal communities (Schadt *et al.*, 2003; Baldrian *et al.*, 2013a). In this study, the activity of extracellular enzymes showed significant seasonal variation, with the highest activity for most enzymes being detected in winter in all horizons but being most pronounced in the litter (Figure 1). This result is not surprising because the fresh litter shed in late autumn contains easily degradable compounds, and its decomposition is rapid over the whole winter period (Šnajdr *et al.*, 2011). Consistent with the high enzyme activities over winter, fungal biomass in litter in the spring increased, with a relative increase in the proportion of non-mycorrhizal taxa (Figure 1).

The litter horizon also exhibited the highest proportion of fungal genera showing seasonal variation. This finding is in agreement with a previous study from a Mediterranean forest, where the extent of

the seasonal variations in microbial communities also decreased with soil depth due to higher seasonal variations in environmental conditions (Andreetta *et al.*, 2012). The changes in the fungal community in the litter were profound. The saprotrophic fungal genera *Mycena*, *Mycosphaerella* and *Naevala* showed 30×, 200× and 350× differences in abundance among seasons. This result partly reflects succession on fresh litter: *Mycosphaerella,* which peaked in autumn, is typical of senescent and freshly fallen oak leaves (Voříšková & Baldrian, 2013), and the other autumn fungal genera, e.g., *Mucor*, *Umbelopsis* and *Lachnellula*, also belong to taxa that grow rapidly in the nutrient-rich environment of fresh litter (Osono, 2006). Additionally, the fungi that increased in winter, *Naevala*, *Rhodotorula* and *Cryptococcus* are typical saprotrophs. These genera were found to be associated with litter decomposition approximately 4 months after abscission (Voříšková & Baldrian, 2013) and thus seem to be supported nutritionally by the last year's litter, which represents, in the ecosystem studied, approximately 40% of the total mass of the L horizon (data not shown). The summer was characterised by a dramatic increase in ectomycorrhizal abundance: compared with spring, the abundance of *Amanita* increased 68×, of *Lactarius* 20× and of *Russula* 7× (Supporting Information Table S2), and the relative abundance of ECM fungi increased from 9 to 29% (Figure 1). The increased abundance of ECM fungi in late summer or autumn has also been previously reported from boreal forests (Wallander *et al.*, 2001; Högberg *et al.*, 2010; Davey *et al.*, 2012).

In the H and Ah horizons, seasonal differences in abundance were recorded for 30% of the dominant taxa. Both horizons exhibited higher enzyme activity during winter. Although in the H horizon this might partly have been due to the priming effect of nutrients leached from litter, the fact that the enzyme activity also increased in the Ah horizon would indicate instead the switch from the use of root-supplied photosynthates to the decomposition of organic matter. This switch was able to maintain a comparable fungal biomass content in spring as well as in winter. Contrary to our expectations, the relative proportion of saprotrophic fungi did not increase during winter-spring as a result of this switch to decomposition. One of the possible explanations might be the temporal switch of certain ECM taxa to a saprotrophic lifestyle, allowing them to preserve their biomass Although the saprotrophic abilities of ECM fungi are still debated (Ekblad *et al.*, 2013), there is a growing evidence derived from enzymatic analyses and in vitro experiments that they are involved in decomposition of litter (Courty *et al.,* 2010; Rineau *et al.,* 2012). This explanation would be in agreement with observations that the enzymatic activities of ECM root tips increased before bud break in oak trees (Courty *et al.*, 2007) and that litter-derived carbon is accummulated by oak roots via ectomycorrhizal mycelia (Bréda *et al.,* 2013). Recent evidence indicates that members of the genus *Russula* contain genes for both exocellulases and ligninolytic peroxidases (Bödeker *et al.*, 2009; Štursová *et al.*, 2012; Voříšková & Baldrian, 2013). Their temporary saprotrophy is quite possible because the relative abundance of this genus during winter was less reduced than that of the other ECM fungi. The fungal biomass content in the Ah horizons increases approximately threefold from spring to summer, which corresponds with the expected increase in photosynthate allocation belowground. The unexpected observation that the proportion of ECM fungi does not increase during this season might indicate that root-supplied carbon can be used by both ECM and by saprotrophs in the soil.

In this study, we demonstrate that the fungal community in a temperate forest soil is very dynamic, showing significant seasonal changes in the activity, biomass

content, composition and relative abundance of different fungal groups. The results showed that the litter community exhibited seasonal changes in composition, while the mineral soil responded rather by changes in fungal abundance. ECM and saprotrophic fungi were indicated as the major players in this respect. Both litter decomposition and photosynthate allocation represent important factors that contribute to the observed seasonal changes. The study was limited to the upper part of the soil profile, which may represent a limitation because it is well established that fungal communities are stratified even much deeper belowground (Rosling *et al.*, 2003). Conclusions about the composition of the whole fungal community can be made, however, since deeper in the soil fungal biomass continues to decrease rapidly (Šnajdr *et al.*, 2008) and the bulk of the community is thus contained within the depth analysed. To achieve a deeper understanding of the seasonal transitions in fungal community functions, it would be necessary, however, to complement the current data with a functional analysis of metatranscriptomes or metaproteomes that would answer important questions about seasonal changes in the physiology of individual taxa, including the extent of mycorrhizal saprotrophy. This study also shows that our understanding of the fungal community composition in those ecosystems where environmental factors show seasonal variation is limited if this phenomenon is not considered.

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Supporting Information

Supporting Information Fig. S1: Principal component analysis of the plot-normalized relative abundances of fungal genera in the H and Ah horizons, seasonal loads and environmental variables.

Supporting Information Table S1: Identification of most abundant fungal OTUs in *Quercus petraea* forest topsoil.

Supporting Information Table S2: Overview of the abundance of fungal genera in *Quercus petraea* forest topsoil by horizons.

IV

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REGULAR ARTICLE

Production of extracellular enzymes and degradation of biopolymers by saprotrophic microfungi from the upper layers of forest soil

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Abstract Production of extracellular enzymes participating in the degradation of biopolymers was studied in 29 strains of nonbasidiomycetous microfungi isolated from *Quercus petraea* forest soil based on the frequency of occurrence. Most of the isolates were ascomycetes and belonged to the genera *Acremonium*, *Alternaria*, *Cladosporium*, *Geomyces*, *Hypocrea*, *Myrothecium*, *Ochrocladosporium*, and *Penicillium* (18 isolates), and two isolates were zygomycetes. Only six isolates showed phenol oxidation activity which was low and none of the strains were able to degrade humic acids. Approximately half of the strains were able to degrade cellulose and all but six degraded chitin. Most strains produced significant amounts of the cellulolytic enzymes cellobiohydrolase and β-glucosidase and the chitinolytic enzymes chitinase, chitobiosidase, and N-acetylglucosaminidase. The highest cellulase activities were found in *Penicillium* strains, and the highest activity of chitinolytic enzymes was found in *Acremonium* sp. The production of the hemicellulose-degrading enzymes α-galactosidase, β-galactosidase, and α-mannosidase was mostly low. The microfungal strains were able to produce significant growth on a range of 41–87, out of 95 simple C-containing substrates tested in a Biolog™ assay, monosaccharides being for all strains the most rapidly metabolized C-sources. Comparison with saprotrophic basidiomycetes from the same environment showed that microfungi have similar cellulolytic capabilities and higher chitinase activities which testifies for their active role in the decomposition of both lignocellulose and dead fungal biomass, important pools of soil carbon.

Keywords Lignocellulose · Soil microfungi · Chitin · Enzymes · Decomposition · Forest biogeochemistry

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Abbreviations

ABTS	2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid
AMC	7-aminomethyl-4-coumarin
CCBAS	Culture Collection of Basidiomycetes
ITS	Internal Transcribed Spacer
MEA	Malt Extract Agar
MnP	Mn-peroxidase
MUF	4-methylumbelliferol

Introduction

Biopolymers contained within the cell walls of plants and fungi represent the major source of carbon in

forest soils entering the environment either with dead plant material during litterfall or as a result of fungal growth. Actually, cellulose, lignin and chitin are the three most abundant biopolymers in terrestrial biomes and the transformation of carbon present within thus represents an important process in the C-cycle. Saprotrophic soil fungi are often considered to be the most efficient decomposers of these biopolymers (Kjoller and Struwe 2002; Baldrian 2008a) and some authors hypothesize that fungi actually dominate certain decomposition niches (de Boer et al. 2005). While this seems to be justified for ligninolytic wood-associated fungi that have been largely characterized due to the biotechnology-related research (Hatakka 2001; Baldrian 2008b) and for the same reason the production of cellulases is well understood in certain fungi, e.g., *Trichoderma* (*Hypocrea*) spp. (Lynd et al. 2002), the abilities of several common groups of soil-inhabiting fungi are not well-characterized in this respect. Only recently, saprotrophic basidiomycetes living in forest soil and litter have been characterized with respect to the production of cellulose, hemicellulose, and lignin-degrading enzymes (Steffen et al. 2000, 2007; Valášková et al. 2007). However, the potential of nonbasidiomycetous microfungi, another important group of soil and litter-associated fungi to attack biopolymers in soil or litter, remains largely unexplored. Unlike ectomycorrhizal fungi, where biodegradative enzyme activities seem to be limited (Baldrian 2009a), several microfungi are typical saprotrophs that produce biopolymer-degrading enzymes (Bhiri et al. 2008; Hayashi et al. 1997; Lynd et al. 2002; Mamma et al. 2008).

In contrast to lignin decomposition which seems to be exclusively performed by saprotrophic basidiomycetes (Baldrian 2008a), degradation of polysaccharides—cellulose, hemicelluloses and chitin—is performed by many nonbasidiomycetous fungi (Chavez et al. 2006; Lynd et al. 2002; Seidl 2008). The degradation of cellulose as both the most abundant and rapidly utilizable biopolymer of plant litter now attracts considerable attention with respect to the understanding of the rates of carbon cycle-related processes (Sinsabaugh et al. 2008). The estimation of cellulose degradation potential by a group of commonly occurring soil microorganisms is thus important. Cellulose degradation is typically performed by the concerted action of three classes of enzymes. Endocellulases cleave in the middle of the cellulose chains while the exocellulases produce cellobiose from either reducing or nonreducing ends. Ultimately, β-glucosidases cleave cellobiose into two glucose molecules (Baldrian and Valášková 2008). However, the ability to produce the individual components of this cellulolytic system varies among cellulolytic fungi (Baldrian and Valášková 2008; Lynd et al. 2002).

Chitin is the most important biopolymer in soils that does not originate in the plant biomass. Full chitin hydrolysis is typically performed by a three component system consisting of endochitinase, chitobiosidase, and N-acetylglucosaminidase. Endochitinases cleave in the middle of the chitin chain while the chitobiosidase (exochitinase) releases chitooligosaccharides from the end of the polymer chain. N-acetylglucosaminidase catalyzes the release of terminal, non-reducing N-acetylglucosamine residues from chitin, with the highest efficiency in cleaving the dimer diacetylchitobiose. Recent genome sequencing data show that the genomes of filamentous fungi typically contain between 10 and 25 different chitinases, compared to only two to four in bacteria (Seidl 2008). This diversity indicates an important role of this group of fungi in chitin transformation in soils as well as the promise for a future use in biotechnology.

The aim of this work was to describe the production of extracellular enzymes by nonbasidiomycetous microfungi isolated from forest soil. Although saprotrophic basidiomycetes are capable to decompose litter more rapidly than nonbasidiomycetous fungi (Osono 2007; Osono and Takeda 2006), litter decomposing in situ differs significantly from the litter decomposed by basidiomycetes only (Valášková et al. 2007). This indicates an important role for nonbasidiomycetous fungi in its degradation. We wanted to answer the following questions:

(1) How do the microfungi potentially contribute to the degradation of cellulose, chitin, and lignin?
(2) What simple carbon compounds are they able to use as substrates for growth? Is the utilization of cellulose- and chitin-derived monomers related to the ability of individual strains to perform cellulose and chitin decomposition?
(3) Can a general conclusion be made on the extracellular enzyme production by nonbasidiomycetous microfungi compared to basidiomycetes?

To answer these questions, we isolated strains of nonbasidiomycetous fungi in the *Quercus petraea*

forest, in the same site where we previously studied litter degradation by isolates of saprotrophic basidiomycetes (Valášková et al. 2007) and where the activity of several enzymes active in biopolymer degradation was detected in situ (Šnajdr et al. 2008).

Materials and methods

Study site, strain isolation and maintenance

The fungi were isolated from an oak (*Quercus petraea*) forest floor (L and H horizons) in the Xaverovský Háj Natural Reserve, near Prague, Czech Republic. The soil was an acidic cambisol (inceptisol) with developed L, H, Ah and A horizons: L—thickness 0.5–1.5 cm, pH 4.3; 46.2% C; 1.76% N; H—thickness 1.5–2.5 cm, pH 3.7; 21.5% C; 0.56% N. High, but spatially variable activity of extracellular ligninolytic and hydrolytic enzymes was found in the soil of that site (Šnajdr et al. 2008) and basidiomycete strains isolated there exhibited production of these enzymes when grown on litter. However, analytical pyrolysis showed significant differences between litter decomposed by isolated basidiomycetes and that decomposed in situ (Valášková et al. 2007).

Samples of forest floor material were collected for isolation of nonbasidiomycete microfungi from early spring to late autumn. Forest floor material was cut into pieces (<2 mm) with sterile scissors and supplemented with sterile distilled water (100 mL water per 1 g of sample wet mass). Serial dilutions (10^{-3} to 10^{-5}) were prepared and plated onto agar plates with modified Smith and Dawson (SD) medium (20 g L^{-1} malt extract, 0.05 g L^{-1} Rose Bengal, 20 g L^{-1} agar, 30 μg L^{-1} streptomycin; Hršelová et al. 1999) and cultivated at 25°C for three to seven days. Single colonies from the plates were re-inoculated onto malt extract agar (MEA) medium (20 g L^{-1} malt extract, 20 g L^{-1} agar). The colonies were selected for isolation based on the frequency of occurrence and colony morphology in order to obtain a representative sample of the most frequently present morphotypes (80 isolates in total). Fungal strains were maintained on MEA at 25°C. For long term strain maintenance, a working collection was established using the Protect™ system (Technical Service Consultants, United Kingdom). Radial growth rate was calculated as the increase of maximal colony diameter during the linear phase of colony extension on MEA medium.

All strains of litter-decomposing basidiomycete fungi (*Collybia maculata* CCBAS 755, *Flammulina velutipes* CCBAS 363, *Gymnopus ocior* CCBAS 287, *Hypholoma fasciculare* CCBAS 283, *Lepista nuda* CCBAS 136, *Marasmius quercophilus* CCBAS 290, *Mycena galopus* CCBAS 139, *Mycena pura* CCBAS 280, *Mycena rubromarginata* CCBAS 299, *Mycena viridimarginata* CCBAS 134, *Pholiota flammans* CCBAS 229, *Rhodocollybia butyracea* CCBAS 286, *Stropharia hornemannii* CCBAS 295, *Stropharia semiglobata* CCBAS 144) were obtained from the Culture Collection of Basidiomycetes of the Institute of Microbiology of the ASCR, v.v.i. (Prague, Czech Republic). The strains CCBAS 280, CCBAS 283, CCBAS 286, and CCBAS 287 were previously isolated from the same site as the microfungi (Valášková et al. 2007), while the other species were selected based on the observations of fruitbodies at the same site or at other *Quercus petraea* forest sites in Central Europe. Fungal strains were maintained on MEA at 25°C.

Identification of strains

The identification of isolated strains was performed using DNA sequencing. DNA was isolated from agar cultures using the Powersoil Kit (MoBio, USA). Isolated genomic DNA was used as a template in PCR reactions using primers for parts of the fungal rDNA region (Gardes and Bruns 1993; White et al. 1990): ITS-1f (5′-CTTGGTCATTTAGAGGAAG TAA-3′) and NL4 (5′-GGTCCGTGTTTCAA GACGG-3′). Each 50 μl reaction mixture contained 5 μl 10× buffer for DyNAzyme DNA Polymeraze (Finnzymes), 3 μl of Purified BSA 100x (10 mg/ml, New England Biolabs), 2 μl of each primer (0.01 mM), 1.6 μl of PCR Nucleotide Mix (10 mM each, Roche), 2 μl polymerase (2U μl^{-1}, DyNAZyme II DNA Polymerase, Finnzymes) and 1 μl of isolated genomic DNA. PCRs were run on TGradient thermocycler (Biometra, Germany), cycling conditions were 1× (95°C 3 min, 55°C 30 s, 72°C 1 min), 30× (95°C 30 s, 55°C 30 s, 72°C 1 min), 1× (95°C 30 s, 55°C 30 s, 72°C 10 min) (Valášková and Baldrian 2009). PCR products were sequenced as a single extension with primer ITS1f by Macrogen Inc. (Korea) using an ABI 3730 XL DNA Analyzer (Applied Biosystems).

Sequences were manually edited and corrected prior to BLAST (blastn) search against the nucle-

otide database at NCBI (http://www.ncbi.nlm.nih.gov/blast). Identification was based on the best blastn match.

Due to low variability of the ITS region in the genus *Penicillium*, new PCR reaction was performed for strains identified as the members of this genus using primers for part of the β-tubulin gene (Glass and Donaldson 1995), Bt2a (5′-GGTAACCAAATCGGT GCTGCTTTC-3′) and Bt2b (5′- ACCCTCA GTGTAGTGACCCTTGGC-3′). PCR was performed using the same protocol as above and the products were sequenced as a single extension with primer Bt2a by Macrogen, Inc. (Korea) using an ABI 3730 XL DNA Analyzer (Applied Biosystems).

For the strains that showed higher than 98% sequence similarity of the rDNA region (or, in the case of *Penicillium* spp., of the β-tubulin gene), one strain representing the >98% similarity group was selected for further analysis. Additional tests of carbon source assimilation patterns and enzyme activity confirmed that the closely related strains were also very similar physiologically (data not shown). This selection yielded 29 representative strains which were further used in the study.

Identification of the sequences was performed with BLAST running against GenBank. Isolates showing higher than 98% similarity with a sequence present in GenBank were assigned to a species level, and other strains were assigned to higher taxa. The identification of strain 62 was performed using the Trichokey online identification tool (http://www.isth.info/tools/molkey/index.php). For all strains, the DNA sequence containing part of the 18S ribosomal RNA gene, the ITS1 region, and part of the 5.8S ribosomal RNA gene has been deposited at GenBank; for the isolates from the genus *Penicillium*, partial sequences of β-tubulin genes were also deposited. The accession numbers are given in Table 1.

Semi-quantitative assay of enzyme production

API ZYM™ (Bio Merieux, France), a laboratory kit for semiquantitative analysis of production of hydrolytic enzymes by microorganisms was used for the comparison of enzyme production in 29 strains of nonbasidiomycetous micromycetes and 14 strains of saprotrophic basidiomycetes. Fungal strains were cultured on MEA medium at 25°C for 14 days. Following incubation, a portion of 1 cm^2 of agar with approximately 7-day-old mycelium was removed, supplemented with 2 mL distilled H_2O, and homogenized with a mortar and pestle. An aliquot of 65 μL of the resulting suspension was then delivered into the API ZYM cupules and incubated at 37°C for 4 h as described in the manufacturer's instructions with slight modifications (de la Cruz et al. 2006). One drop each of ZYM A (25 g Tris-hydroxymethyl-aminomethane, 11 mL 37% HCl, 10 g sodium lauryl sulfate, 100 mL H_2O) and ZYM B (0.12 g Fast Blue BB, 50 mL methanol, 50 mL dimethyl sulfoxide) reagents were added to the cupules. The color reactions were read after 5 min of incubation and compared with the color code provided by the manufacturer. Results were recorded as zero (zero nanomoles substrate hydrolysed), 1 (5 nanomoles substrate hydrolysed), 2 (10 nanomoles substrate hydrolysed), 3 (20 nanomoles substrate hydrolysed), 4 (30 nanomoles substrate hydrolysed), or 5 (≥40 nanomoles substrate hydrolysed).

Production of extracellular enzymes

Agar plate screening was performed as described by Steffen et al. (2000) using Petri dishes (90 mm, 25 mL/plate) containing MEA supplemented with (1) 0.25 g L^{-1} 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) for the detection of laccase (phenoloxidase) activity, (2) 0.1 g L^{-1} $MnCl_2$. $4H_2O$ for Mn-peroxidase activity or (3) 1 g L^{-1} of humic acid to assay the humic acid decolorization potential. Humic acid was prepared from the H horizon material of the site of strain isolation as previously described (Valášková et al. 2007). Plates were inoculated with a 7-mm agar plug containing fresh mycelium from an MEA culture and incubated at 25°C. After two weeks, ABTS plates were examined for the formation of green rings around the fungal mycelia, indicating the presence of extracellular radical-generating enzymes (laccase, peroxidases). Mn plates were evaluated after six weeks of incubation for the formation of black and dark brown spots of MnO_2 caused by the action of Mn-peroxidase. Humic acid plates were examined after six weeks for bleaching, indicating the transformation of humic acids into low molecular mass fulvic acids.

Enzymes were extracted from fungal cultures growing on MEA medium. Five 0.5 cm×0.5 cm squares were cut from agar plates below 7-day-old mycelium, cut into small pieces, mixed with 50 mM

sodium acetate buffer, pH 5.0 (3 mL cm^{-2}), and extracted for 2 h at 4°C with constant mixing. Filtered extracts were used for analyses.

The activities of β-glucosidase, cellobiohydrolase, N-acetylglucosaminidase, chitobiosidase, endochitinase, α-glucosidase, β-xylosidase, phosphomonoesterase, phosphodiesterase, and arylsulfatase in the extracts were assessed with 4-methylumbelliferyl(MUF)-β-D-glucopyranoside, MUF-β-D-cellobioside, MUF-N-acetyl-β-D-glucosaminide, MUF-N,N'-chitobioside, MUF-N,N', N"-chitotrioside, MUF-α-D-glucopyranoside, MUF-β-D-xylopyranoside, MUF phosphate, bis-(MUF) phosphate, and MUF sulfate, respectively. Alanine- and leucine aminopeptidases were assayed with L-alanine-7-amido-4-methylcoumarin and L-leucine-7-amido-4-methylcoumarin. Fluorescence of the released reaction products was measured as previously described (Baldrian 2009b) using a method modified from Vepsäläinen et al. (2001). Substrates (100 µL in DMSO) to give the final concentration of 500 µM were combined with three technical replicates of 100 µL of extracts in a 96-well multiwell plate. For the background fluorescence measurement, 100 µL of 50 mM sodium acetate buffer, pH 5.0 were combined with 100 µL of 4-methylumbelliferol or 7-aminomethyl-4-coumarin standards to correct the results for fluorescence quenching (Vepsäläinen et al. 2001). The multiwell plates were incubated at 40°C, and fluorescence was recorded from 5 min to 125 min using the Infinite microplate reader (TECAN, Austria), using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The quantitative enzymatic activities after blank subtraction were calculated based on standard curves of 4-methylumbelliferone and 7-amido-4-methylcoumarin. One unit of enzyme activity was defined as the amount of enzyme forming 1 nmol of 4-methylumbelliferone and 7-amido-4-methylcoumarin liberated per min and was expressed per square area of fungal culture.

Laccase activity in extracts was measured (but not detected) by monitoring the oxidation of ABTS in citrate-phosphate (100 mM citrate, 200 mM phosphate) buffer (pH 5.0) at 420 nm according to previous methods (Bourbonnais and Paice 1990). The activities of Mn-peroxidase and lignin peroxidase were not measured because plate assays of Mn^{2+} oxidation and humic acid bleaching were negative and because the presence of these enzymes is not anticipated in nonbasidiomycetous fungi (Morgenstern et al. 2008).

Degradation of cellulose and chitin was tested by methods modified from Smith (1977) and Untereiner and Malloch (1999). Briefly, 6 mL of basal medium (1.25 g L^{-1} KH_2PO_4, 0.625 g L^{-1} $MgSO_4$. $7H_2O$, 15 g L^{-1} agar) in 15-mL test tubes was overlaid with 0.6 mL of an overlay (2 g L^{-1} malt extract, 4.38 g L^{-1} cellulose azure or chitin azure, 15 g L^{-1} agar). The tubes were inoculated by 7-mm agar plugs with mycelia pre-cultivated on ME agar plates. Polysaccharide degradation was monitored for 28 d as the release of color into the basal medium and scored as negative (−; no color change), positive (+; light blue to blue color), and strongly positive (++; violet color). All enzyme assays were performed in at least three biological replicates.

Analysis of carbon source assimilation patterns

Carbon source utilization by fungal isolates was examined using the BIOLOG Phenotype MicroArrays as described by Druzhinina et al. (2006). Fungal cultures were initially grown on 2% (w/v) Malt Extract Agar until sporulation occurred. Inoculum was prepared by rolling a sterile, wetted cotton swab over the conidia-bearing colony and suspending it in 10 ml sterile phytagel solution (0.25% phytagel, 0.03% Tween 40) in disposable test tubes (15× 120 mm). The spore density was adjusted to 75±2% transmission at 590 nm wavelength. An aliquot of 90 µL spore suspension was then dispensed into each well of a presterilized Biolog FF MicroPlate (BIOLOG, USA) which contained 95 wells of different prefilled substrates plus one well with no substrate as a control. The inoculated microtiter plates were incubated in the dark at 25°C and the mycelial growth was measured after intervals of 24, 48, 72, 96, and 168 h as the absorbance at 750 nm using the Infinite microplate reader (Tecan, Austria). Three biological replicates were run for each fungal isolate.

Analysis of the Phenotype MicroArray was performed on the absorbance measured at 750 nm, where the values are directly proportional to mycelial density. The data from the 72 h reading were used since growth limitation and/or sporulation in wells occurred during later readings. The data were corrected for initial absorbance at time 0. To obtain the relative substrate utilization, data were divided by average well color development, i.e., the mean absorbance in all wells of the plate. The ability to

Table 1 Identification and selected properties of microfungal isolates. Cellulose and chitin degradation were measured as dye release from an azo-stained polymeric substrate, laccase activity was measured as ABTS oxidation on plates with MEA medium, and the activity of other enzymes was measured using the Api Zym test. Metabolic diversities were calculated from carbon source utilization data obtained with BIOLOG FF plates, and radial growth rates were measured on MEA plates

Strain	Identification	Genbank ITS	Genbank β-tubulin	Closest hit[a]	Similarity (%)	Genbank closest hit	Cellulose degradation	Chitin degradation	Laccase	Alkaline phosphatase	Esterase	Esterase Lipase	Leucine arylaminidase
02	*Penicillium* sp.	FJ379818	FJ387170	*Penicillium decaturense*	96	EF198558.1	–	+	–	3	0	0	0
03	*Penicillium* sp.	FJ379826	FJ387194	*Penicillium* sp.	96	EF198559.1	–	–	–	3	1	0	1
04	*Acremonium* sp.	FJ379830		*Acremonium* sp.	99	EF577238.1	+	+	+	3	1	1	2
05	*Penicillium* sp.	FJ379811	FJ387183	*Penicillium citreonigrum*	91	FJ904908.1	–	+	–	3	1	1	0
07	*Penicillium* sp.	FJ379813	FJ387178	*Penicillium* sp.	95	DQ834933.1	+	++	–	5	2	1	1
09	*Geomyces pannorum*	FJ379798		*Geomyces pannorum*	98	DQ189225.1	+	+	–	4	1	0	1
12	*Penicillium* sp.	FJ379812	FJ387184	*Penicillium citreonigrum*	90	FJ904908.1	–	+	–	4	1	2	0
13	*Penicillium* sp.	FJ379825	FJ387177	*Penicillium decaturense*	86	EF198558.1	–	+	–	2	1	1	0
14	*Penicillium coprobium*	FJ379802	FJ387185	*Penicillium coprobium*	99	AY674425.1	+	–	–	3	0	0	3
15	*Penicillium* sp.	FJ379815	FJ387180	*Penicillium angulare*	86	EF198563.1	+	+	–	0	1	0	0
16	*Myrothecium inundatum*	FJ379829		*Myrothecium inundatum*	99	AJ302005.1	–	+	+	1	0	0	0
18	*Penicillium* sp.	FJ379809	FJ387190	*Penicillium griseolum*	81	EF506213.1	+	+	+	5	2	1	1
19	*Umbelopsis* sp.	FJ379795		*Umbelopsis ramanniana*	90	AB193542.1	+	++	–	4	1	0	2
20	*Penicillium* sp.	FJ379814	FJ387179	*Penicillium* sp.	90	DQ834933.1	+	–	–	3	2	1	1
24	*Penicillium* sp.	FJ379805	FJ387187	*Penicillium canescens*	96	DQ658166.1	+	++	–	0	0	0	0
26	*Cladosporium cladosporoides*	FJ379801		*Cladosporium cladosporoides*	99	AY251074.2	+	+	+	2	1	1	4
30	*Penicillium glandicola*	FJ379803	FJ387186	*Penicillium glandicola*	99	AY674414.1	–	–	–	2	0	0	1
36	*Ochrocladosporium* sp.	FJ379832		*Ochrocladosporium frigidarii*	96	FJ755255.1	+	+	+	2	2	2	0
39	*Ochrocladosporium frigidarii*	FJ379833		*Ochrocladosporium frigidarii*	98	FJ755255.1	–	+	+	2	0	0	1
40	*Alternaria tenuissima*	FJ379834		*Alternaria tenuissima*	98	AY154712.1	+	–	–	3	1	2	4
41	*Penicillium soppii*	FJ379804	FJ387188	*Penicillium soppii*	100	DQ285613.1	–	+	–	2	0	0	1
43	*Penicillium glabrum*	FJ379807	FJ387192	*Penicillium glabrum*	100	EF198547.1	+	+	–	2	1	1	0
50	*Penicillium glabrum*	FJ379808	FJ387193	*Penicillium glabrum*	97	EU128587.1	–	+	–	2	1	1	0
54	*Penicillium* sp.	FJ379824	FJ387176	*Penicillium decaturense*	92	EF198558.1	–	+	–	2	0	0	1
61	*Penicillium bialowiesense*	FJ379806	FJ387189	*Penicillium bialowiesense*	99	AY674440.1	+	–	–	2	1	0	3
62	*Hypocrea semiorbis*	FJ379831		*Hypocrea semiorbis*	–		++	+	–	2	1	1	2
66	*Penicillium* sp.	FJ379828	FJ387196	*Penicillium* sp.	94	FJ619265.1	–	+	–	4	1	1	3
67	*Mucoromycotina* sp.	FJ379796		*Mucoromycotina* sp.	98	GQ241272.1	–	++	–	1	0	0	1
71	*Dothideomycetes* sp.	FJ379835		*Dothideomycetes* sp.	90	GQ153107.1	–	+	–	0	2	0	0

Strain	Valine arylaminidase	Acid phosphatase	α-Galactosidase	β-Galactosidase	β-Glucuronidase	α-Glucosidase	β-Glucosidase	N-acetylglucosaminidase	α-Mannosidase	α-Fucosidase	Metabolic diversity[b,c]	Radial extension rate (μm h^{-1})
02	0	4	0	0	0	2	3	3	0	0	87	46
03	0	5	2	1	0	2	3	4	0	0	62	74
04	1	5	0	1	0	2	3	4	0	0	70	199
05	0	5	2	1	0	2	3	5	1	0	60	130
07	0	5	1	1	0	2	3	5	1	0	86	202
09	0	3	0	0	0	1	2	3	0	0	79	58
12	0	5	3	2	0	3	4	4	1	2	62	116
13	0	4	1	1	0	2	2	3	0	0	83	79
14	0	3	2	0	0	0	2	2	1	0	59	106
15	0	5	2	0	0	0	2	5	0	0	74	130
16	0	3	0	0	0	0	1	0	1	0	n.d.	95
18	0	4	2	2	0	3	3	4	0	0	44	40
19	1	1	0	0	0	2	0	2	0	0	n.d.	167
20	0	5	2	3	0	2	5	5	0	1	n.d.	204
24	0	3	1	0	0	0	1	4	0	0	73	149
26	0	4	1	1	1	4	3	0	0	0	81	196
30	0	2	0	0	0	0	5	3	0	0	41	65
36	0	5	3	0	0	4	3	5	0	0	n.d.	48
39	0	5	0	0	0	1	2	4	0	0	74	54
40	3	2	3	4	0	5	4	5	0	0	n.d.	281
41	0	3	0	2	1	1	2	4	0	0	n.d.	107
43	0	5	3	1	0	2	3	4	0	2	79	146
50	0	5	2	1	0	3	2	5	0	2	75	135
54	0	4	0	0	0	1	1	3	0	0	78	125
61	0	4	2	3	0	1	2	5	0	1	58	38
62	0	2	1	2	0	0	0	1	0	0	n.d.	375
66	1	5	1	1	0	3	2	5	2	0	79	333
67	0	3	2	2	0	0	2	3	0	0	n.d.	240
71	0	0	0	0	0	3	2	0	0	0	n.d.	102

[a] Identification of *Penicillia* spp. was based on the β-tubulin sequence, that of *Hypocrea* sp. on the Trichokey online identification tool, (http://www.isth.info/tools/molkey/index.php), and that of all other isolates on the rDNA (ITS) sequence

[b] The number of simple carbon substrates from the FF Biolog pool able to utilize the substrate for growth (max. = 95)

[c] n.d.: not determined; growth tests were impossible to perform due to little or no sporulation

grow on a given substrate was defined as the ability to cause a 1.5× higher increase in the absorbance of the substrate well with respect to the control well.

Statistics

Statistical tests were conducted using the software package Statistica 7 (StatSoft, USA). Differences between groups were tested by a one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Principal component analysis was used to analyze the variability of enzyme production among saprotrophic basidiomycetes and microfungi. Source data for the analysis were the values of the API ZYM test plus the data from laccase and MnP activity from agar plate screening (activity present or absent). In all cases, differences at $P<0.05$ were regarded as statistically significant.

Results

Identification of isolates

A relatively rich nutrient medium supplemented with Rose Bengal as an inhibitor of bacterial growth was used for isolation of microfungi in order to enrich for fast growing nonbasidiomycetous species. Based on the rDNA region containing the ITS sequence, most of the isolates were identified as members of the genus *Penicillium* (*Eurotiales*, *Ascomycota*). Isolates belonging to the genus *Penicillium*, were identified based on a partial sequence of the β-tubulin gene. Out of the total of 18 sequences, six were assigned to a specific species (Table 1). The other ascomycete strains belonged to *Hypocreales* (the genera *Acremonium*, *Hypocrea* and *Myrothecium*), *Pleosporales* (*Alternaria* and *Ochrocladosporium*), *Capnodiales* (*Cladosporium*), *Leotiomycetes* (*Geomyces*), and *Dothideomycetes*. Two strains were zygomycetes, with one belonging to *Mucorales* (*Umbelopsis*) (Table 1). None of the isolates obtained by this method belonged to *Basidiomycota*. The radial extension rate of all strains was relatively fast, at 40–375 μm h^{-1} (Table 1).

Enzyme production by soil microfungi

Degradation of cellulose was detectable in 15 of the 29 strains, but was slow for all strains except strain 62 (Table 1). All but five strains were able to produce cellobiohydrolase (exocellulase). All isolates except strain 62 were able to produce β-glucosidase (Fig. 1) and also exhibited fast growth on cellobiose. Strains 07, 18, 24, and 39 grew on this substrate at the same rate as on glucose or even faster. Interestingly, the production of endoglucanase was frequently accompanied by little or no cellobiohydrolase activity, and, vice versa, several strains with high exocellulase production did not produce endocellulase. Strains with high cellobiohydrolase activity also exhibited high β-glucosidase activity ($P<0.001$) .

These isolates had a low potential to act in lignin degradation or the transformation of phenols. None of the strains were able to either oxidize Mn^{2+} or decolorize humic acids. Only six strains exhibited some potential to oxidize ABTS (Table 1). All of these strains were also tested for phenoloxidase production in liquid culture with glucose or cellulose as carbon sources, but did not produce detectable activity (data not shown).

The potential to degrade chitin was widespread among the isolates. Twenty strains were able to degrade azo-dyed chitin, with strains 07, 19, 24, and 67 showing the fastest degradation. The same number of strains was also able to produce endochitinase in MEA medium, 22 strains produced chitobiosidase, and all isolates produced N-acetylglucosaminidase, although the level of production varied highly (Fig. 1); the best producers of chitobiosidase were strains 04 and 12 with 255 and 287 nmol min^{-1} cm^{-2}, and strain 12 also exhibited the highest endochitinase activity (280 nmol min^{-1} cm^{-2}). All strains were able to grow rapidly on N-acetylglucosamine, the product of N-acetylglucosaminidase action (Fig. 2); the growth rates of strains 02, 07, 09, 13, 18, and 54 were comparable on N-acetylglucosamine and on glucose, with both faster than on cellobiose. Interestingly, the production of N-acetylglucosaminidase negatively correlated with the growth rate on N-acetylglucosamine ($P=0.047$).

Enzymes degrading hemicelluloses were produced by all tested strains, but the production of individual enzymes differed widely. When grown on MEA, all but three strains (14, 19, and 62) produced β-xylosidase, but the activities were usually 5–10× lower than that of β-glucosidase; the activity of the two hydrolases correlated strongly ($P<0.001$). α-Galactosidase was produced by 18 strains and β-

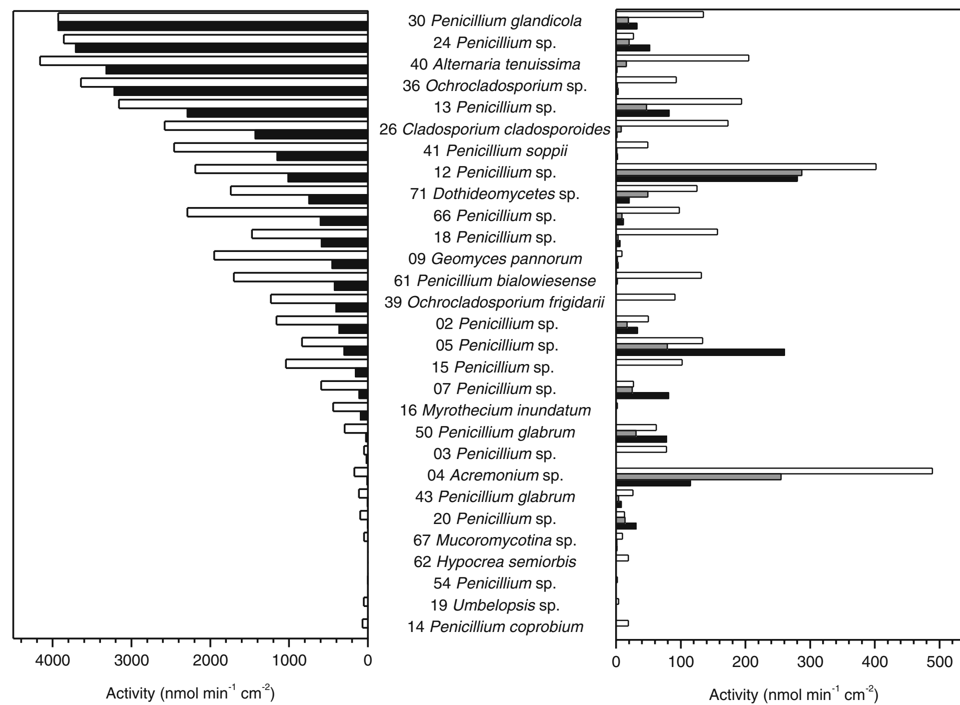


Fig. 1 Activity of cellulolytic and chitinolytic enzymes of fungal isolates in 7-day-old mycelia on MEA medium. Left panel: activity of β-glucosidase (*open bars*) and cellobiohydrolase (*black bars*); right panel: activity of N-acetylglucosaminidase (*open bars*), chitobiosidase (*gray bars*), and endochitinase (*black bars*). These data represent the means of three replicates. Standard errors (less than 30%) were omitted for clarity

galactosidase by 17 strains, but α-mannosidase, α-fucosidase, and β-glucuronidase were produced only by 7, 5, and 2 strains, respectively, with all showing low activities (Table 1).

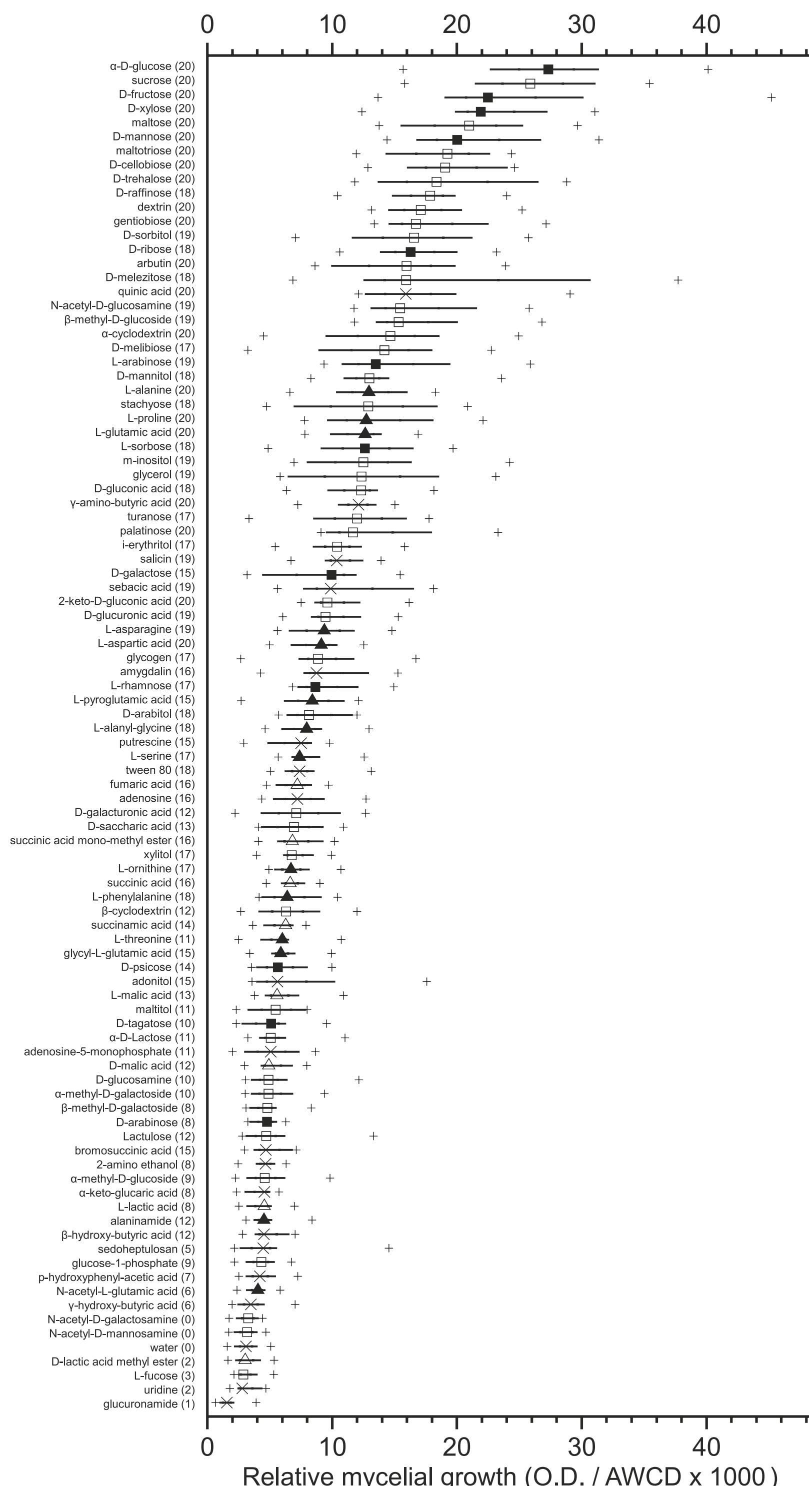
Among the other enzymes tested, acid phosphatase was produced by 28 strains, α-glucosidase (amylase) by 22, phosphodiesterase by 21, leucine arylaminidase by 18, arylsulfatase by 15, lipase by 14, and valine arylaminidase by four strains. Only strain 09 produced alanine and leucine aminopeptidases; both were produced at high levels showing that they may be important to this fungus.

In the comparison of activities of extracellular hydrolytic enzymes included in the Api Zym test and the ligninolytic enzymes laccase and Mn-peroxidase among microfungi isolated in this work and saprotrophic basidiomycetes occurring in the same habitat, substantial differences were found that clearly separated these two groups (Fig. 3). Basidiomycete fungi exhibited significantly higher activities of ligninolytic enzymes: Mn-peroxidase was produced exclusively by basidiomycetes, and laccase was produced by all tested basidiomycete strains but only by six strains of microfungi. Basidiomycetes also showed higher activities of esterase, lipase, leucine and valine arylaminidases, α-mannosidase and β-glucuronidase. Microfungi, on the other hand, exhibited significantly higher N-acetylglucosaminidase activity.

Utilization of simple carbon substrates as growth resources

Utilization of simple carbon substrates was tested using the BIOLOG FF system containing 95 different carbon substrates (Fig. 2). The analysis was performed for 20 strains where the sporulation makes it possible to prepare inoculum of a defined initial biomass concentration. The metabolic diversity, i.e., the number of substrates from the set where a strain was able to produce significant growth, ranged from 41 to 87, with 65% of strains able to grow on 70 or more substrates (Table 1). In general, monosacchar-

Fig. 2 Carbon source utilization by fungal isolates based on absorbance data (turbidity) at 750 nm following incubation of the BIOLOG FF Microplates at 25°C for 72 h. Turbidity data measure mycelial growth, indicative of substrate utilization. The data are based on the mean absorbance after growth on individual carbon substrates relative to average well color development (AWCD). Medians are represented by large symbols that indicate substrate type (*full squares*: monosaccharides; *open squares*: oligosaccharides or saccharide derivatives; *full triangles*: amino-acids or derivatives; *open triangles*: simple organic acids; *crosses*: other compounds). Interquantile ranges are represented by *horizontal lines* and Q_{10} and Q_{90} by *small crosses*. Numbers in parentheses for each substrate indicate how many strains (n=20) were able to grow significantly on the substrate (absorbance increase at least 1.5× higher than that in water)



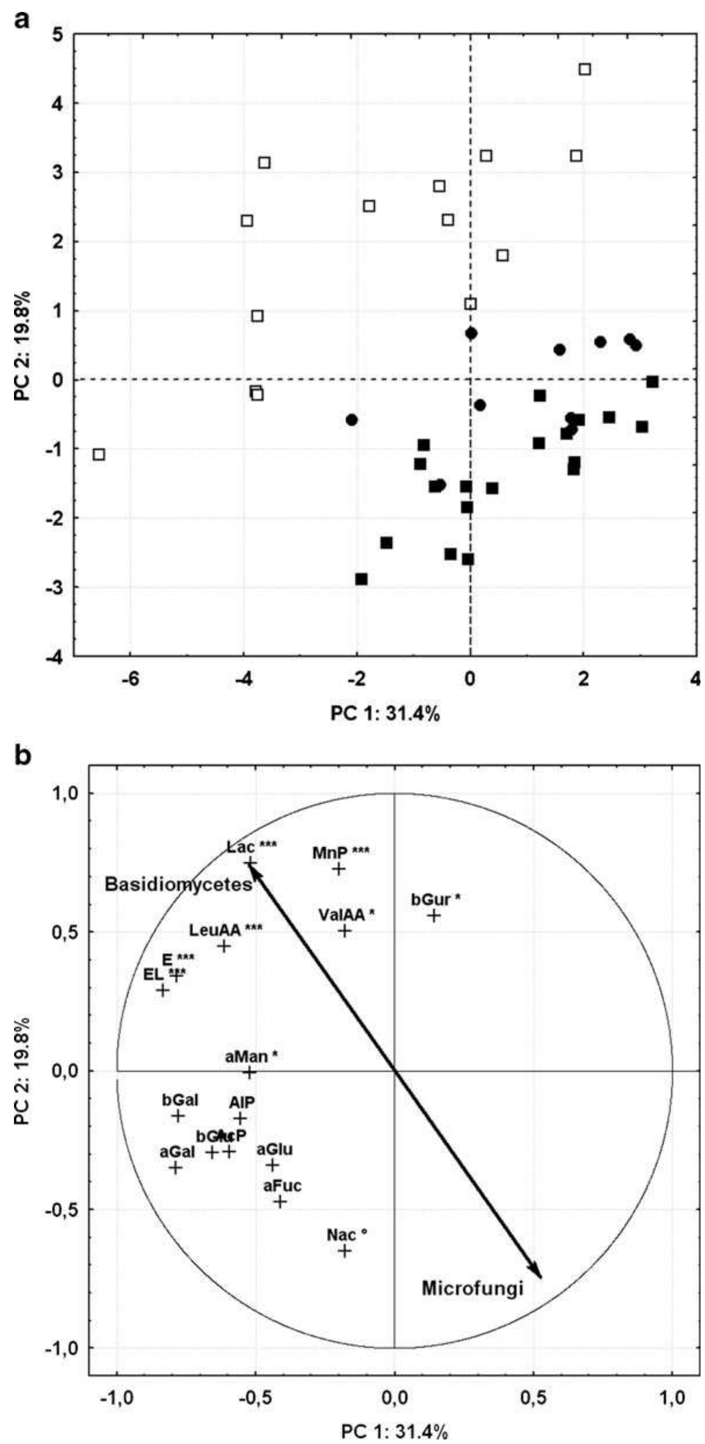


Fig. 3 Principal component analysis of the activities of selected extracellular enzymes produced by fungi inhabiting *Quercus* sp. forest topsoil. Panel A: Principal component loads of individual fungal strains; *open symbols*: saprotrophic basidiomycetes; *full squares*: *Penicillium* strains, *full circles*: other microfungal strains. Panel B: component loads of individual enzymes and fungal groups. Abbreviations: AcP: acid phosphatase; aFuc: α-fucosidase; aGal: α-galactosidase; aGlu: α-glucosidase; AlP: alkaline phosphatase; aMan: α-mannosidase; bGal: β-galactosidase; bGlu: β-glucosidase; bGur: β-glucuronidase; E: esterase; EL: esterase lipase; Lac: laccase; LeuAA: leucine arylamidase; MnP: Mn-peroxidase; Nac: N-acetylglucosaminidase; ValAA: valine arylamidase; *asterisks* indicate significantly higher activity in saprotrophic basidiomycetes than in microfungi (* $P<0.05$, ** $P<0.01$; *** $P<0.001$); *circles* indicate significantly higher activity in microfungi ($P<0.05$)

ides were the most rapidly metabolized group of substrates. One notable exception was arabinose, one of the major monosaccharide components of oak litter, which was only utilized by eight strains, and which only supported very slow growth (Fig. 2). Several disaccharides and saccharide derivatives were also commonly used by microfungi. Among amino acids, alanine, glutamic acid, and proline were utilized by all strains and supported rapid growth. Organic acids usually supported only slow growth.

Discussion

Soil microfungi, taxonomically belonging to ascomycetes and zygomycetes, represent an important group of fungi in forest soils. Based on DNA analyses, ascomycetes represented approximately 50% of the total fungal community in the L horizon and approx. 25% in the H horizon in the soil used in this study (Voříšková et al. 2009). In the L and H horizons, *Ascomycota* may represent between 30–60% of the total fungal community, and *Zygomycota* up to 5% (O'Brien et al. 2005). In beech litter, 85–97% of clones belonged to ascomycetes, with the rest mainly zygomycetes (Aneja et al. 2006). In this study, members of the genera *Acremonium*, *Alternaria*, *Cladosporium*, *Geomyces*, *Hypocrea*, *Myrothecium*, *Ochrocladosporium*, *Penicillium*, and *Umbelopsis* as well as two unidentified clones of *Mucoromycotina* and *Dothideomycetes* were isolated from the L and H horizons of *Quercus petraea* soil. More than half of the isolates belonged to the genus *Penicillium*. *Acremonium*, *Cladosporium*, *Geomyces*, *Mucor*, *Penicillium*, and *Trichoderma* have been repeatedly isolated from both forest and agricultural soils (De Bellis et al. 2007; Grishkan 1996; Keller and Bidochka 1998). *Penicillium*, *Trichoderma*, and *Myrothecium* were the most common genera of ascomycetes revealed by DNA sequencing in beech litter (Aneja et al. 2006), demonstrating that the fungi isolated in this study represent the total microfungal community relatively well.

We detected neither Mn^{2+} oxidation or humic acid degradation in the studied strains which indicates the lack of ligninolytic peroxidases. The low production of Mn-peroxidase by nonbasidiomycetous microfungi reported by (Řezáčová et al. 2006) is most likely due to inaccurate methodology since the presence of this enzyme seems to be limited to certain basidiomycete genera (Morgenstern et al. 2008). Slow ABTS oxidation was found in agar plates with *Myrothecium inundatum*, *Acremonium* sp., *Ochrocladosporium*, *Cladosporium* and one *Pennicillium* strain, and the low or missing activity on ABTS is sharply contrasting with the high activity in all tested basidiomycete strains. ABTS oxidation by *Myrothecium verrucaria* and probably also by *Acremonium murorum* was probably due to bilirubin oxidase, another Cu-containing oxidase (Baldrian 2006; Hoegger et al. 2006). *Cladosporium cladosporoides* and a *Penicillium* strain were also previously reported to degrade lignin-like compounds (Claus and Filip 1998; Zheng et al. 1999) or humic acids (Gramss et al. 1999; Paul and Mathur 1967), however, the identity of the enzymes involved remains unclear.

The inability of microfungi to degrade lignin may also be the reason for the slower litter transformation by microfungi than by basidiomycetes. When decomposition of *Fagus crenata* was compared for different species of fungi, the loss of dry mass caused by cord-forming basidiomycetes was 15–57%, and by *Xylariaceous* ascomycetes it was 4–14% (Osono and Takeda 2002). These strains were able to bleach the decomposed litter, indicating polyphenol transformation. On the other hand, no bleaching activity was detected in other ascomycete and zygomycete species (including the genera *Acremonium*, *Cladosporium*, *Penicillium*, *Trichoderma*, and *Mucor*), and the mass loss was typically between 1% and 5% (Osono and Takeda 2002). Similar results were obtained with *Betula* litter (Osono and Takeda 2006). In the same study, only less than 10% of carbohydrates were mineralized by *Penicillium glabrum* and *Trichoderma viride* compared to 15–60% in basidiomycetes (Osono and Takeda 2006). This indicates that ascomycetes are likely able to utilize just a limited fraction of the easily accessible compounds in litter and not even cause significant polysaccharide degradation.

This study indicates that limited production of hemicellulose-degrading enzymes may be another explanation for lower utilization of polysaccharides by nonbasidiomycetous microfungi. Activities of α-mannosidase and β-glucuronidase were low in all microfungi, and only a few of our isolates, most notably *Alternaria tenuissima*, were able to produce significant amounts of hemicellulases (Table 1). This is in contrast to the relatively high production of hemicellulases by saprotrophic basidiomycetes on

lignocellulose, reported previously (Baldrian et al. 2005; Steffen et al. 2007; Valášková et al. 2007).

Most isolates were able to produce cellobiohydrolase and β-glucosidase, often in high quantities. Strains with high cellobiohydrolase activities also exhibited high β-glucosidase activities ($P<0.001$). Approximately half of the strains were also able to produce endocellulase and seem to possess the complete system for cellulose hydrolysis. The production of endocellulase was frequently accompanied by low or no cellobiohydrolase activity (strains 04, 14, 19, 20, 43, and 62). This may indicate that these strains are primarily oriented towards the degradation of cellulose-containing cell walls in order to get access into cells rather than to use the cellulose as a source of carbon and energy. Interestingly, one of these strains was identified as *Hypocrea semiorbis*, belonging to the genus where cellobiohydrolases are usually produced in high titers and whose members possess an extensive set of cellulolytic enzymes; *Hypocrea jecorina* whole genome sequence contains two genes encoding cellobiohydrolases and eight genes in five families of endoglucanases (Martinez et al. 2008). It was previously reported that *Penicillium* sp., *Acremonium* sp., and *Trichoderma* sp. isolates from a subtropical forest degraded cellulose (but not lignin) (Hao et al. 2006), and the production of endocellulase was also detected in several *Penicillium* and *Trichoderma* strains from grasslands (Daynes et al. 2008; Deacon et al. 2006). The presence of one or more cellulolytic enzymes was reported from individual members of the genera *Acremonium, Cladosporium, Mucor, Myrothecium, Penicillium*, and *Trichoderma* (Bhiri et al. 2008; Ikeda et al. 2007; Skare et al. 1975; Somkuti 1974; Whitaker 1951). However, our results show that the production levels of these enzymes vary greatly among strains of the same genus. The best producers of cellobiohydrolase and β-glucosidase were found among *Penicillium* strains, and this genus may thus be an attractive target for future biotechnological exploration.

Chitinases have been previously purified from *Mucor*, *Myrothecium*, *Penicillium*, and *Trichoderma* (de la Cruz et al. 1992; Rast et al. 1991; Vyas and Deshpande 1989). In a study on grassland microfungi, only a few chitin degraders were recorded (Deacon et al. 2006). Using the same method, we demonstrated that chitin degradation is widespread among microfungi isolated from forest soil: most isolates were able to degrade chitin, with the best producer being the *Acremonium* isolate. Despite the fact that production of chitinolytic enzymes was demonstrated in several wood-decomposing basidiomycetes (Lindahl and Finlay 2006), our results show that microfungi were significantly better N-acetylglucosaminidase producers than litter-decomposing basidiomycetes. There was a strong correlation between the production of endochitinase, chitobiosidase, and N-acetylglucosaminidase ($P\leq0.001$). The fact that the production of N-acetylglucosaminidase negatively correlated with the growth rate on N-acetylglucosamine might indicate that the species hydrolyzing chitin are using the enzyme to cross the cell wall of fungi or invertebrates to get to more readily utilizable compounds rather than using it as a growth source. The general use of N-acetylglucosamine as a C source strongly contrasts with the fact that none of the isolates were able to grow on N-acetylgalactosamine and N-acetylmannosamine, amino-sugars potentially providing comparable amounts of carbon, nitrogen and energy. Microfungi turned out to be metabolically versatile, with the ability to use many different C sources.

With respect to the growth rates on individual substrates, strains can be divided into two main groups: (1) isolates growing rapidly on glucose but significantly slower on most other substrates (e.g., the mono- and oligosaccharides derived from cellulose, hemicelluloses and chitin), and (2) strains growing rapidly on many different substrates, often at rates similar or even slightly higher than on glucose. This division is supported by the fact that strains growing faster on cellobiose than on glucose also grew faster on N-acetylglucosamine ($P<0.001$).

The most important components of cellulose, hemicelluloses and chitin (cellobiose, xylose, and N-acetylglucosamine) supported rapid growth of most strains. The comparison of taxonomic similarity (ITS or β-tubulin sequence similarity) and the metabolic profiles showed that the most closely related strains (i.e., the pairs 05/12, 43/50 and 03/66 with >95% sequence identities) also showed similar carbon utilization patterns. However, this finding was not valid at greater taxonomic distances, and the genus *Penicillium* was metabolically variable, probably due to niche differentiation.

Our results show that soil microfungi differ from saprotrophic cord-forming basidiomycetes in their abilities to decompose soil biopolymers. They are not likely to play an important role in lignin

degradation. On the other hand, several strains are able to produce cellulolytic enzymes, and the production of chitinases seems to be widespread and higher than in basidiomycetes. Forest soil microfungi can thus actively participate in the transformation of both litter and dead fungal biomass, both belonging to the richest carbon sources of forest soils.

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Chemical composition of litter affects the growth and enzyme production by the saprotrophic basidiomycete *Hypholoma fasciculare*

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ABSTRACT

Chemical composition of litter has previously been reported to affect *in situ* decomposition. To identify its effects on a single species level, the saprotrophic basidiomycete *Hypholoma fasciculare* was grown on 11 types of litter with variable chemical composition (N content of 3.4–28.9 mg g^{-1}), and the mass loss of litter and lignin, production of extracellular enzymes and fungal biomass were followed. After 12 weeks, mass loss ranged from 16 % to 34 %. During early decomposition stages, litter mass loss, fungal biomass production (estimated by ergosterol content) as well as fungal substrate use efficiency all increased with increasing initial N content of the litter. The initial litter decomposition rate was significantly positively correlated with the activities of arylsulfatase, cellobiohydrolase, endoxylanase and phosphatase. Contrary to expectations, the lignin content did not affect litter mass loss, when covariation with N content was accounted for. The ratio of lignin loss to total mass loss depended on the litter type and did not reflect the activities of ligninolytic enzymes.

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Introduction

Leaf litter together with photosynthesis-derived carbon in rhizodeposition are the main sources of organic matter in hardwood forest soils (Bray & Gorham 1964; Litton *et al.* 2003). Because a large amount of small-molecular-mass compounds is withdrawn from leaves during senescence, the remaining plant cell wall biopolymers, cellulose, hemicelluloses and lignin represent the most important resources for soil decomposer microorganisms present in leaf litter. Among microorganisms, fungi are regarded as major decomposers of lignocellulose and especially its polymeric fractions in the soil (Kjoller & Struwe 2002; Baldrian 2008), and among fungi, saprotrophic basidiomycetes represent an important group whose members efficiently decompose both the plant cell wall polysaccharides and lignin (Baldrian 2009a; Šnajdr *et al.* 2010; Baldrian *et al.* 2011). This is due to the fact that these fungi produce a wide variety of extracellular enzymes involved in the decomposition of lignocellulose biopolymers (Steffen *et al.* 2000; Steffen *et al.* 2007; Valášková *et al.* 2007). Among the

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extracellular enzymes produced by fungi, polysaccharide hydrolases decomposing cellulose (endocellulase, cellobiohydrolase, and β-glucosidase), hemicelluloses (endoxylanase and β-xylosidase) and other polysaccharides (α-glucosidase and N-acetylglucosaminidase), deliver C and energy for the growth of mycelium, while ligninolytic enzymes (laccase and peroxidases) are considered responsible for liberating polysaccharides (mainly hemicelluloses) from covalent complexes with lignin (Baldrian 2008; Baldrian & Valášková 2008; Thevenot *et al.* 2010). The supply of macronutrients to the growing mycelium is provided by the action of hydrolytic enzymes acting on organic N, P and S-containing molecules (phosphatases, arylsulfatase, peptidases (Baldrian 2009b)).

Dead plant biomass – litter and wood – is characterised by the relative excess of C and relatively low N and P contents. As such, it is more suitable for decomposition by fungi than by bacteria. Indeed, highest fungal biomass in forest soils is associated with litter layer with the highest C/N ratio while the share of bacteria increases with soil depth (Berg *et al.* 1998; Šnajdr *et al.* 2008). The initial stages of litter decomposition in particular are dominated by fungi (Šnajdr *et al.* 2011). Cord-forming basidiomycetes are often claimed as particularly effective decomposers of N-limited lignocellulose litter due to their ability to potentially translocate N from nutrient-rich patches using their mycelial systems (Boddy 1999) which allow them to decrease the N-limitation.

Decomposition of litter under natural conditions, however, is a process involving not only basidiomycetes, but complex communities composed of various fungal and bacterial taxa that change during succession (Šnajdr *et al.* 2011; Kjoller & Struwe 2002; Osono 2007). Although some fungal decomposers are generally present in different litter types, the successional series of dominant fungal species are litter-type specific (Frankland 1998; Koide *et al.* 2005; Osono & Takeda 2006), as are also the rates and limit values of decomposition of different litter types (Berg 2000; Berg *et al.* 2010). One of the proposed explanations of these observations is based on the assumption that content of (or limitation by) nutrients, especially N and P, and susceptibility to decomposition (lignin content) are the major determinants of fungal community composition, and consequently the decomposition rates (Treseder 2008; Gusewell & Gessner 2009). However, since the differences in litter composition also imply differences in microbial communities, it is difficult to find the relative role of individual chemical quality parameters on decomposition. The use of a single species in a decomposition test provides an experimental tool to assess the relationships between litter chemistry and decomposition without the effects of temporary changes in microbial community composition. Furthermore, the identification of the physiological responses of saprotrophic basidiomycetes to litter composition can help to understand the factors that might potentially promote or limit their dominance or competitive success in a given substratum.

Activity of extracellular enzymes is frequently used as a proxy of decomposition. It remains unclear, however, which of the extracellular enzymes are the most important for fungal litter decomposition and consequently define the overall rate of decomposition in general. Recent results show that hemicelluloses are decomposed faster than cellulose and lignin, at least in some types of litter (Šnajdr *et al.* 2011) and cellulose and hemicellulose-decomposing enzymes are thus likely candidates for the rate-limiting determinants of litter decomposition.

While most studies of litter decomposition consider mass loss or enzyme activities, few quantify the production of fungal mycelia in different types of litter, largely all due to the technical limitations of fungal biomass measurements in complex substrata, e.g. by ergosterol measurements. The efficiency of litter transformation into fungal biomass is, however, of considerable importance as high biomass yields allow a fungus to invest into exploratory growth of mycelial cords while low yields will likely result in energy-dependent survival only. Only a few papers have reported on fungal biomass (expressed mostly as ergosterol content) in terrestrial environments (Conway *et al.* 2000; Sinsabaugh *et al.* 2002; Gusewell & Gessner 2009; Šnajdr *et al.* 2011) and all of these studies consider decomposition by mixed populations of both bacteria and fungi. In single-species studies, chitin has been previously used as a proxy of fungal biomass production (Boberg *et al.* 2008), but ergosterol measurements, may contribute to the comparison of results from single-species and complex microcosm studies.

The aim of this paper was to identify how chemical composition of litter affects its decomposition by a saprotrophic basidiomycete in terms of rates of the mass and lignin loss, the production of extracellular enzymes and formation of fungal biomass. We hypothesized that the content of lignin in litter would cause high production of ligninolytic oxidases and peroxidases to increase the availability of carbohydrates while a high N content would inhibit lignin decomposition. To test this, the saprotrophic basidiomycete *Hypholoma fasciculare* was grown on 11 types of litter differing in their chemical composition, and the mass loss of litter and specifically lignin, production of extracellular enzymes and formation of fungal biomass were followed as well, as changes in the available C and N compounds, during decomposition. *H. fasciculare* is a wood-associated saprotroph which also exploits litter and performs slow and nonselective lignin decomposition and mineralization (Valášková *et al.* 2007; Šnajdr *et al.* 2010). Mycelia of *H. fasciculare* are frequently found in soils of forests with different dominant tree species and its growth strategy in topsoil where it produces exploitative mycelial systems (Kampichler *et al.* 2004) points to extensive use of litter-derived nutrients during explorative growth. The fungus may thus serves as a model saprotroph capable of utilizing lignocellulose from both wood and litter.

Materials and methods

Fungal strains, materials and cultivation

The saprotrophic basidiomycete *H. fasciculare* CCBAS 281 was obtained from the CCBAS collection (Institute of Microbiology of the ASCR, v.v.i., Prague, Czech Republic). The strain was originally isolated from a fruit body collected in an oak (*Quercus petraea*) forest in the Xaverovský Háj Natural Reserve, near Prague, Czech Republic (Valášková *et al.* 2007). For preparation of inocula, the fungus was grown in the dark at 25 °C for 14 d in ME medium ($20\ g\ l^{-1}$ malt extract, $15\ g\ l^{-1}$ agar). Mycelial agar plugs (7 mm in diameter) were cut from the edge of an actively growing colony and used as inocula.

Litter of 11 tree species (*Alnus glutinosa*, *Betula pendula*, *Fagus sylvatica*, *Larix decidua*, *Picea abies*, *Pinus sylvestris*, *Pinus nigra*, *Populus tremula*, *Q. petraea*, *Salix caprea* and *Tilia cordata*) was collected from the forest floor immediately after abscission in the Sokolov region, Czech Republic, and air-dried before use.

The cultivation of fungi was performed in 100-ml Erlenmeyer flasks containing 5 g air-dried ground litter (particle size > 10 mm), corresponding to 4.50 g dry mass (85 °C). The litter was moistened with 22.5 ml distilled water (final water content 83 %). The flasks were autoclaved (2×30 min at 121 °C) and inoculated with two agar plugs of mycelium. The cultures were incubated at 25 °C in the dark. For enzyme analysis, three flasks of each litter type were collected each sampling time (weeks 2, 4, 6, 8, 10 and 12). For the analysis of fungal biomass content, three flasks of each litter type were collected after 4, 8 and 12 weeks of incubation. Three flasks per litter type were collected at the beginning of the experiment and after 4, 8 and 12 weeks of incubation, for the analysis of litter chemistry.

Litter analysis

Dry mass content was assessed by drying litter at 85 °C to a constant mass and dry litter was milled before chemical analyses. Klason lignin content was measured as dry weight of solids after hydrolysis with 72 % (w/w) H_2SO_4 (Kirk & Obst 1988). Analysis of litter chemical composition was performed as described previously (Šantrůčková *et al.* 2006). Available phosphorus extractable with oxalate (Pox) was determined by extraction of 0.5 g of litter with 50 ml of acidic ammonium oxalate solution (0.2 M $H_2C_2O_4$ + 0.2 M $(NH_4)_2$ C_2O_4 at pH 3). Water-extractable compounds were extracted from litter step by step in cold water (water:litter, 10:1, v/w, 30 min at 20 °C) and hot water (water:litter, 10:1, v/w, 16 hr at 80 °C). Extractable C and N were then determined on a TOC analyser (Skalar FormacsHT, the Netherlands), and NH_4 and NO_3 using flow injection analyser (Foss Tecator 5042, Sweden). The elemental analyses (C, H and N) were performed for the initial litter and for litter after decomposition (Perkin–Elmer 2400, MA, USA). All measurements were performed in duplicates for each flask and mean values were used for statistical evaluation. All results are expressed on actual dry mass basis.

Quantification of fungal biomass

Ergosterol was used as a marker of fungal biomass. Total ergosterol was extracted and analysed according to Nylund & Wallander (1992). Mycelial samples from liquid culture (0.5 g) were sonicated with 3 ml 10 % KOH in methanol at 70 °C for 90 min. Distilled water (1 ml) was added and the samples were extracted three times with 2 ml cyclohexane, evaporated under nitrogen, redissolved in methanol and analysed isocratically using a Waters Alliance HPLC system (Waters, USA) with methanol as a mobile phase at a flow rate of 1 ml min^{-1}. Ergosterol was quantified by UV detection at 282 nm.

Whole samples of fungal cultures on litter (~4 g dry mass) were sonicated with 30 ml of 10 % KOH in methanol and 10 ml of cyclohexane at 70 °C for 90 min in Pyrex flasks. After sonication, 10 ml of distilled water and 15 ml of cyclohexane were added and the mixtures were shaken for 15 min and sonicated until phase separation occurred. The supernatants were collected, the cultures were twice re-extracted with 15 ml of cyclohexane, as mentioned above, and supernatants from all extractions were combined. After extraction, samples were dried at 55 °C until constant mass. The mass of the added KOH was subtracted from the total dry mass. The solvent was evaporated using a vacuum pump RVO 200A (INGOS s.r.o., Czech Republic) and the residua after evaporation were redissolved in 5 ml of methanol. The dissolved samples (1 ml) were transferred to 1.5 ml microtubes, centrifugated for 3 min at 6 000$\times g$, and the supernatant was used as a sample for HPLC.

To determine the relationship between ergosterol content and fungal biomass in cultures on litter, *H. fasciculare* was also cultivated on liquid medium, and the dry mass of fungal mycelium and its ergosterol content were quantified. *H. fasciculare* was grown in 250 ml Erlenmeyer flasks containing 40 ml of ME medium. Flasks were inoculated with two agar plugs and incubated at 25 °C in the dark. To obtain mycelia from exponential, early stationary and late stationary phase, flasks were sampled after 7, 14 and 21 d of incubation (five replicates). Since the ergosterol content in mycelia of different growth stages did not show big differences (1 364 $\pm$ 526, 1 609 $\pm$ 637, and 1 161 $\pm$ 396 μg/g of dry fungal mycelia after 7, 14 and 21 d, respectively), the mean value of 1 379 μg ergosterol/g *H. fasciculare* mycelium was used for calculations. Substrate use efficiency was calculated as a ratio of fungal biomass after the experiment and the mass of litter transformed into fungal biomass + the mass of mineralized litter.

Enzyme assays

At each sampling, the cultures in Erlenmeyer flasks were cut into small pieces and soaked with 40 ml distilled water. The homogenized substrata were extracted at 4 °C for 2 hr on a shaker. Extracts were filtered through Whatman 5 filter paper and the filtrates were kept frozen at −18 °C until analysis by spectrophotometry.

Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation of ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) in citrate–phosphate (100 mM citrate, 200 mM phosphate) buffer (pH 5.0) at 420 nm (Bourbonnais & Paice 1990). Manganese peroxidase (MnP, EC 1.11.1.13) activity was assayed in succinate–lactate buffer (100 mM, pH 4.5) according to (Ngo & Lenhoff 1980). MBTH (3-methyl-2-benzothiazolinone hydrazone) and DMAB (3,3-dimethylaminobenzoic acid) were oxidatively coupled by the enzymes, and the resulting purple indamine dye was detected spectrophotometrically at 595 nm. The results were corrected by the activities of the samples without manganese (for MnP) – the addition of manganese sulphate was substituted by an equimolar amount of ethylenediaminetetraacetate (EDTA). Other ligninolytic peroxidases are not produced by the studied fungal strain and were thus not measured (Valášková *et al.* 2007).

Endo-1,4-β-glucanase (endocellulase, EC 3.2.1.4) and endo-1,4-β-xylanase (EC 3.2.1.8) activities were measured with azo-dyed carbohydrate substrates (carboxymethyl cellulose and birchwood xylan, respectively) as described previously

(Valášková et al. 2007). The reaction mixture contained 0.2 ml of 2 % dyed substrate in 200 mM sodium acetate buffer (pH 5.0), and 0.2 ml sample. The reaction mixture was incubated at 40 °C for 60 min and the reaction was stopped by adding 1 ml of ethanol followed by 10 s vortexing and 10 min centrifugation (10 000×*g*). The amount of released dye was measured at 595 nm and the enzyme activity was calculated according to standard curves correlating the dye release with the release of reducing sugars.

Cellobiohydrolase (EC 3.2.1.91) activity was assayed in microplates using *p*-nitrophenyl-β-D-cellobioside (PNPC). The reaction mixture contained 0.16 ml of 1.2 mM PNPC in 50 mM sodium acetate buffer (pH 5.0) and 0.04 ml sample. Reaction mixtures were incubated at 40 °C for 90–120 min. The reaction was stopped by adding 0.1 ml of 0.5 M sodium carbonate, and absorbance was measured at 400 nm. Activities of 1,4-β-glucosidase (EC 3.2.1.21), 1,4-β-xylosidase (EC 3.2.1.37) and 1,4-β-N-acetylglucosaminidase (chitinase; EC 3.2.1.52) were assayed using *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-β-D-xyloside and *p*-nitrophenyl-N-acetyl-β-D-glucosaminide respectively, using the same method (Valášková et al. 2007). Activities of acidic phosphatase (EC 3.1.3.1) and arylsulfatase (EC 3.1.6.1) were assayed using 2 g l^{-1} *p*-nitrophenylphosphate and 50 mM *p*-nitrophenylsulfate in the same buffer (Baldrian 2009b).

All spectrophotometric measurements were made using a microplate reader (Sunrise, Tecan, Switzerland) or a UV–VIS spectrophotometer (Lambda 11, Perkin–Elmer, MA, USA). For the comparison of values obtained in a system with changing water contents and dry mass, enzyme activities were expressed per g of original dry mass of litter. One unit of enzyme activity was defined as the amount of enzyme forming 1 μmol of reaction product/min.

Statistical tests were conducted using the software package Statistica 7 (StatSoft, USA). Statistical significance of differences among treatments was evaluated using one-way ANOVA and Pearson's correlation coefficients and *t*-values were calculated for linear regressions. Differences and correlations at $P \leq 0.05$ were regarded as statistically significant. Microcal Origin 7.0 (Microcal, USA) was used to test the quality of linear and nonlinear curve fits and to obtain the fit parameters.

Results

Chemical analysis of fresh litter showed that content of N varied widely among litters ranging from 3.4 in *P. abies* up to 28.9 mg g^{-1} in *A. glutinosa* (Table 1). Litters also varied in the amount of extractable phosphorus ranging between 0.06 and 0.59 mg g^{-1}. *F. sylvatica* and *L. decidua* litters exhibited the highest Klason lignin content. Generally, lignin content of litter decreased with increasing N content, and the lowest lignin content was found in the N-richest litter of *A. glutinosa* (Table 1). The extractable fractions of total C and N differed among litter types. Most of the extractable N was present in the organic form; the mineral NH_4 and NO_3 fractions represented from 7 % to 53 % depending on litter type. At the end of the experiment, organic fractions of extractable N largely exceeded the mineral ones in all litter types, accounting for more than 90 % of extractable N; the only exception was *A. glutinosa* where the organic fraction accounted for 36 % only (Supplementary Table 1). This indicates efficient utilization of mineral N by the fungus in most litter types.

The initial rate of substratum decay by *H. fasciculare* during the first 2 weeks of the experiment was slow in all types of litter but accelerated later. Within 12 weeks the fungus caused the highest mass lost in *A. glutinosa* and *L. decidua* litter (34 %), while the *T. cordata*, *P. abies* and *P. nigra* litters were the slowest to decompose (Fig 1). The ratio of Klason lignin loss to total mass loss depended on the litter type; preferential lignin decomposition was found in five litter types while preferential decomposition of non-lignin structures was observed in the others (Fig 2).

The content of ergosterol after 8 weeks incubation ranged from 29 μg g^{-1} in the *P. abies* litter to 213 μg g^{-1} in *P. tremula* litter (Fig 1). With the exception of *P. nigra* litter where fungal biomass increased during the whole experiment, other litter types exhibited a peak of ergosterol content at 4 weeks (*A. glutinosa*) or 8 weeks (all the other litters), followed by a more or less steep decrease. When the ergosterol/biomass ratio of 1 379 μg ergosterol per g fungal biomass was used, the amount of transformed litter could be defined as the sum of

Table 1 – Initial chemical composition of litter and loss of litter dry mass during 12-week incubation with *Hypholoma fasciculare*. Data represent averages and standard errors of means

Litter	Total C (mg/g)	Extractable C (%)	Total N (mg/g)	Extractable N organic (%)	Extractable N (NH_4–N) (%)	Extractable N (NO_3–N) (%)	P_{ox}[a] (mg/g)	C/N[b] week 0	C/N[b] week 12	Klason lignin (mg/g)	Loss of dry mass (%)
Alnus glutinosa	471 ± 2	17.7	28.9 ± 0.8	13.7	2.82	0.07	0.28 ± 0.01	16.3	10.3	337 ± 1	34.3 ± 0.5
Betula pendula	493 ± 1	15.8	7.9 ± 0.1	15.8	1.94	0.24	0.59 ± 0.01	62.7	39.4	507 ± 14	28.5 ± 1.0
Fagus sylvatica	477 ± 1	10.4	6.5 ± 0.3	14.0	2.98	0.39	0.18 ± 0.01	72.9	53.1	567 ± 14	24.2 ± 1.5
Larix decidua	481 ± 1	16.3	3.4 ± 0.0	7.4	3.19	0.64	0.44 ± 0.01	141.3	89.8	549 ± 18	32.9 ± 1.2
Picea abies	472 ± 1	11.1	4.1 ± 0.0	5.0	2.50	0.21	0.06 ± 0.01	114.0	94.6	447 ± 8	16.3 ± 0.5
Pinus nigra	505 ± 1	6.6	6.5 ± 0.1	5.6	0.47	0.18	0.12 ± 0.00	77.9	58.5	534 ± 8	16.5 ± 1.1
Pinus sylvestris	501 ± 1	7.3	5.4 ± 0.2	5.2	2.21	0.13	0.12 ± 0.00	92.0	71.1	512 ± 11	22.3 ± 0.4
Populus tremula	463 ± 2	20.9	17.5 ± 0.8	16.5	3.51	0.25	0.36 ± 0.03	26.4	17.7	418 ± 30	27.9 ± 0.9
Quercus petraea	467 ± 2	15.6	13.5 ± 0.2	12.7	1.78	0.20	0.38 ± 0.01	34.6	22.4	434 ± 27	22.9 ± 0.5
Salix caprea	463 ± 2	6.3	8.7 ± 0.2	5.3	2.08	0.14	0.54 ± 0.01	53.0	34.9	441 ± 14	19.0 ± 0.5
Tilia cordata	458 ± 2	13.3	8.0 ± 0.2	11.7	2.17	0.28	0.43 ± 0.00	57.2	45.3	462 ± 30	15.9 ± 0.5

a Pox: available phosphorus (extractable with oxalate).
b C/N ratio is based on total C and N content.

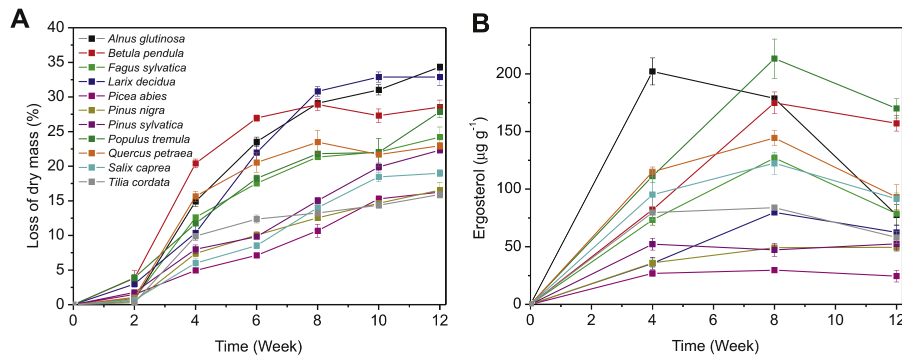

Fig 1 – Loss of dry mass (A) and ergosterol content (B) during a 12-week incubation of the saprotrophic basidiomycete *Hypholoma fasciculare* on different litters. The data represent mean ± SEM ($n = 3$).

the litter mass lost and the litter mass incorporated into fungal biomass. Considering this, transformation of litter went on in all litter types until week 8 when 13–38 % of litter was transformed (the minimum and maximum in *P. abies* and *A. glutinosa*). During weeks 8–12, substantial litter transformation only continued in the slowly decomposing litters (*P. abies*, *P. nigra*, *Pinus sylvatica*, *S. caprea* and *T. cordata*) while the transformation of the faster decomposing litters slowed down substantially. The observed loss of mass between 8 and 12 weeks in these litters occurred at the

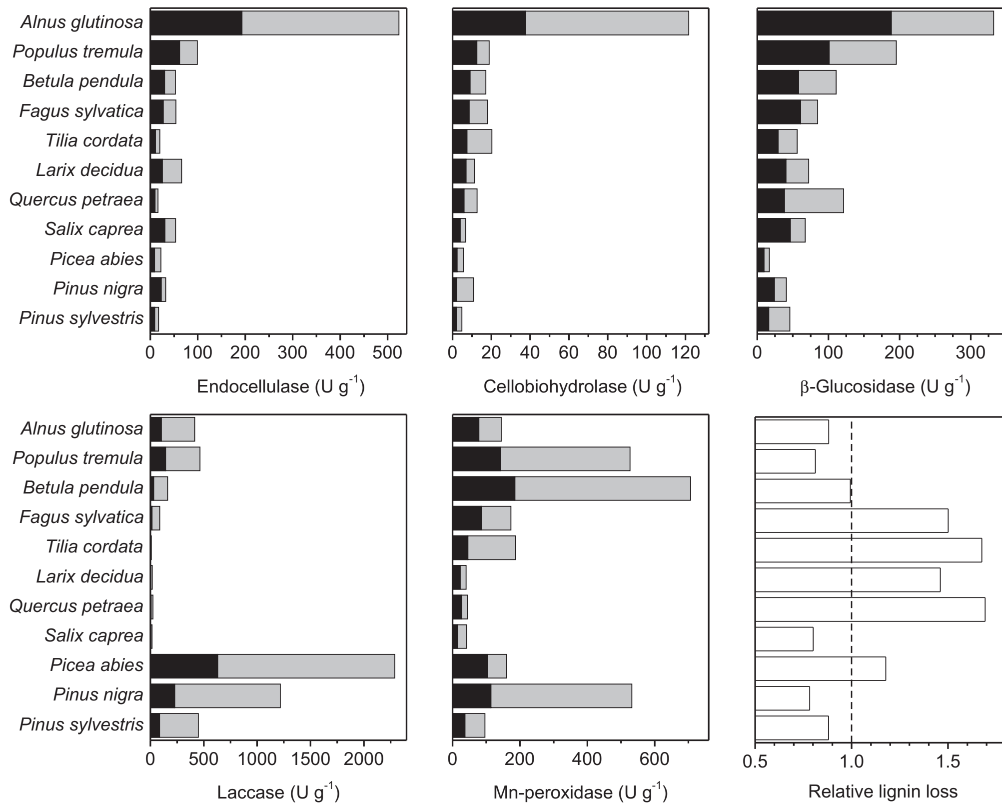

Fig 2 – Activities of selected extracellular enzymes during a 12-week incubation of the saprotrophic basidiomycete *Hypholoma fasciculare* on different litters and the ratio of lignin mass loss/total mass loss at the end of the experiment. Black bars represent mean activities over the 12-week period, gray bars represent peak activities.

expense of the fungal biomass. At week 12, litter transformation ranged from 18 % in *P. abies* to 38 % in *A. glutinosa*.

At the beginning of the experiment, there was an exponential relationship between total C/N of the litter and C/N in water-extractable fraction. At the end of the experiment, this relationship was linear, due to the fact that the C/N in the water-extractable fraction decreased, most likely due to the preferential mineralization of the water-extractable carbon fraction (Fig 3).

Since the chemical composition of litter (Klason lignin content, polysaccharide content and composition) changed during the course of decomposition, and some of its components were likely rapidly depleted from the litter, the effect of litter chemical composition on enzyme production, fungal biomass production and the relationships between loss of dry mass and enzyme activities were also tested over the initial decomposition period (until 4 weeks). Ergosterol content at 4 weeks was positively correlated with the initial content of total N in litter ($P = 0.000$; Fig 4). Highest mass loss was from the litters with a high initial N content (Fig 3). Ergosterol content also negatively correlated with the initial content of lignin in litter ($P = 0.004$). However, this was due to the fact that Klason lignin content in litter decreased with increasing N content. A composite linear model showed that N content in litter explained 74 % of the variation in ergosterol production, and the further effect of lignin in the model was quantitatively insignificant. Thus, N content but not lignin content was important for determining fungal growth. Substrate use efficiency increased with the increasing initial litter N content with the exception of *A. glutinosa* litter, where fungal biomass content peaked early and decreased substantially until 12 weeks (Fig 3).

H. fasciculare produced all measured extracellular enzymes in all types of litter. The activity of the enzymes, however, differed substantially among litters (Fig 2; Table 2). The activity of laccase in *P. abies* litter was distinctively higher than in the rest of litter types, while the highest activity of Mn-peroxidase was recorded in *B. pendula*, *P. tremula*, and *P. nigra* litter. In contrast to that, laccase activity was negligible in *T. cordata* litter and Mn-peroxidase was low in *S. caprea*, *L. decidua* and *Q. petraea* litter. Activities of hydrolytic enzymes participating in the decomposition of cellulose and hemicelluloses were highly correlated with each other and also with the activities of N-acetylglucosaminidase and phosphatase. This was particularly apparent for endocellulase, cellobiohydrolase and β-glucosidase; enzymes participating in the decomposition of cellulose (Fig 2). The highest mean activities of endo-1,4-β-glucanase, endo-1,4-β-xylanase, cellobiohydrolase, β-glucosidase, β-xylosidase, N-acetylglucosaminidase and acid phosphatase were detected in *A. glutinosa* litter (Fig 2; Table 2).

Enzyme activities showed similar time courses of activity in individual litter types (Supplementary Table 1). The activity of ligninolytic enzymes Mn-peroxidase and especially laccase changed substantially with time, both enzymes being active mainly during the first 4 weeks (Supplementary Table 1). Also acid phosphatase was most active during the initial phases of decomposition. The activity of endocellulase was initially low but increased later. For all the other enzymes, no general temporal trends of activity were observed.

The activity of four extracellular enzymes − arylsulfatase ($P = 0.001$), cellobiohydrolase ($P = 0.015$), endoxylanase ($P = 0.044$) and acid phosphatase ($P = 0.044$) − were significantly correlated with mass loss in the first 4 weeks (Fig 4), but the best

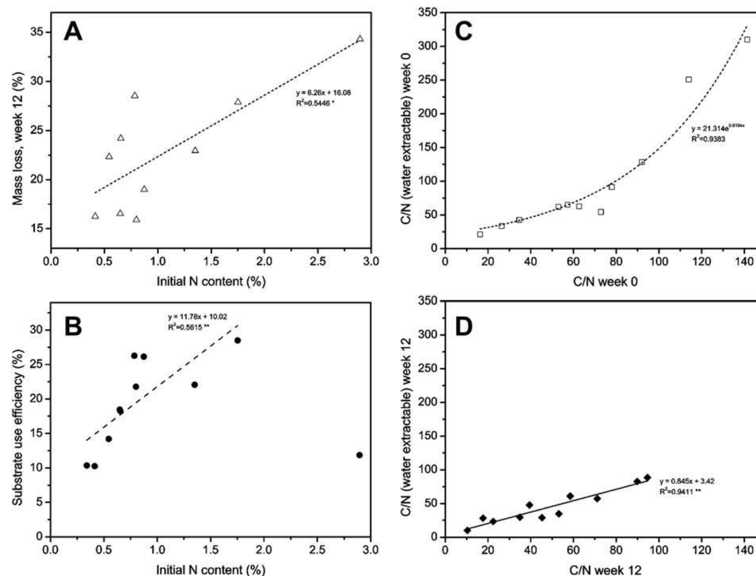

Fig 3 − Relationships between initial N content, mass loss and substrate use efficiency (the proportion of decomposed substrate allocated to fungal biomass production) during a 12-week incubation of the saprotrophic basidiomycete *Hypholoma fasciculare* on different litters. Correlation of initial N content and mass loss (A); initial N content and substrate use efficiency (B). Correlations of C/N ratio in total litter and in extractable litter fraction before the experiment (C) and at the end of the experiment (D). Asterisks indicate statistical significance of linear correlations (*$P \le 0.05$, **$P \le 0.001$).

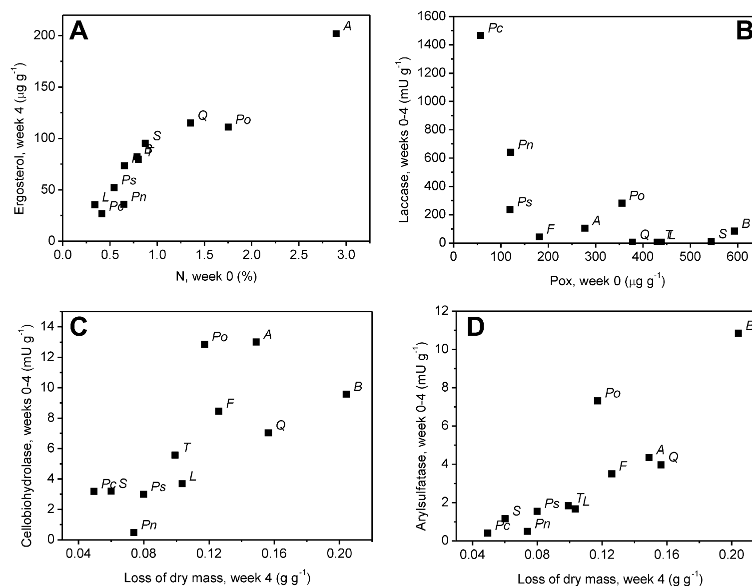

Fig 4 – Relationships among chemical parameters of litter (content of total N and available P), fungal biomass contents and activities of extracellular enzymes during a 12-week incubation of the saprotrophic basidiomycete *Hypholoma fasciculare* on different litters. Litters: A – *Alnus glutinosa*, B – *Betula pendula*, F – *Fagus sylvatica*, L – *Larix decidua*, Pc – *Picea abies*, Pn – *Pinus nigra*, Po – *Populus tremula*, Ps – *Pinus sylvestris*, Q – *Quercus petraea*, S – *Salix caprea*, T – *Tilia cordata*.

predictor of enzyme activity was the total N concentration in litter, which was closely correlated with activities of all hydrolytic enzymes except endoxylanase and arylsulfatase. Production of ligninolytic enzymes was significantly affected only by Pox, which decreased laccase activity ($P = 0.027$; Fig 4). The relationships between mass loss after 12 weeks and litter composition were generally weak. It seems that the litter chemistry affected the rates of enzyme production and fungal growth rather than the limit values for decomposition: the mass loss of *L. decidua* litter after 12 weeks was similar to that of *A. glutinosa*, despite the fact that *L. decidua* litter exhibited the second highest lignin content and the lowest N content, while *A. glutinosa* litter contained the most N and the least lignin.

Discussion

The chemical composition of dead litter, which varies among plant species, is known to affect rates of decomposition (Hättenschwiler & Gasser 2005). In our study, mass loss in different litters ranged between 16–34 % after 12 weeks of incubation with *H. fasciculare*. These values are comparable to those obtained with other saprotrophic basidiomycetes: 19–44 % in birch litter after 3 months in the presence of *Mycena* and *Collybia* species (Osono & Takeda 2006) or several litter-decomposing fungi on *Q. petraea* litter (Steffen *et al.* 2007; Valášková *et al.* 2007). Previous observations suggested that

Table 2 – Mean activities of extracellular enzymes during a 12-week cultivation of the saprotrophic basidiomycetes *Hypholoma fasciculare* on different litters

Litter	Endoxylanase ($U\ g^{-1}$)	β-xylosidase ($U\ g^{-1}$)	N-acetylglucosaminidase ($U\ g^{-1}$)	Acidic phosphatase ($U\ g^{-1}$)	Arylsulfatase ($U\ g^{-1}$)
Alnus glutinosa	130.3	35.2	204	486	4.3
Betula pendula	34.7	9.4	78	155	7.9
Fagus sylvatica	20.1	10.1	72	90	3.0
Larix decidua	4.4	6.0	79	101	5.3
Picea abies	4.4	1.7	16	34	1.0
Pinus nigra	7.0	6.7	68	86	2.1
Pinus sylvestris	7.0	2.3	61	70	2.1
Populus tremula	69.7	13.0	106	254	9.6
Quercus petraea	9.1	7.8	51	139	2.2
Salix caprea	31.1	3.9	63	85	1.8
Tilia cordata	1.1	8.4	32	64	2.6

coniferous litters decompose *in situ* more slowly than litters from broadleaved trees (Miyamoto *et al.* 2000; Osono & Takeda 2002; Osono *et al.* 2003; Osono & Takeda 2006) pointing at potential differences among these groups of litters. Here we show that this rule does not apply for single-species decomposition by *H. fasciculare*: the coniferous litters of *P. sylvestris* and especially *L. decidua* exhibited high mass losses while the decomposition of *T. cordata* litter was slow.

A. glutinosa litter had distinctively higher N content and extractable C and N compared to the other litter types. The contents of extractable N or C did not decrease significantly with time and thus did not limit nutrient availability during decomposition. Total N content was the best predictor of fungal biomass production in the beginning of the experiment. This is in contrast with earlier observations on synthetic media, where both organic and mineral N inhibited the growth of certain saprotrophic basidiomycetes (Keyser *et al.* 1978) but in agreement with a previous study on *Mycena epipterygia* on *Pinus* litter, where N addition increased substrate use efficiency and C addition had no effect (Boberg *et al.* 2008). This observation reflects the fact that C/N ratio in the fungal biomass is typically much lower than in most litters: typically between 8 and 25 (Koide & Malcolm 2009).

Ergosterol content has been claimed to be an unreliable estimator of fungal biomass in environmental samples (Mille-Lindblom *et al.* 2004), based on the observation that ergosterol added to samples as pure compound or as dead fungal biomass decomposes slowly. Here we show that within a living fungal mycelium, ergosterol concentrations may change rapidly, increasing with biomass accumulation and decreasing during senescence. In addition to litter mass loss, a significant part of the litter – up to >30 % of the decomposed litter mass – can be transformed into fungal biomass which is in agreement with *in situ* studies (Frankland *et al.* 1978; Frey *et al.* 2003). The effect of litter quality on the content of microbial biomass was also confirmed in an *in situ* decomposition study showing 4–15× higher biomass of soil organisms on ash litter compared to beech (Bjornlund & Christensen 2005). The ability of *H. fasciculare* to mineralize litters with low N content in our closed system shows that its decomposition can be achieved even without incorporation of external N by translocation – the mechanism proposed to compensate for N deficiency (Frey *et al.* 2003).

We have demonstrated that initial loss of dry mass significantly correlated with the activities of arylsulfatase, cellobiohydrolase, endoxylanase and phosphatase. Among these, cellobiohydrolase is the best candidate for the rate-limiting enzyme of fungal decomposition, since it catalyses the rate-limiting step of cellulose decomposition (Baldrian & Valášková 2008), while arylsulfatase and phosphatase activities most likely correspond to the rate of decomposition by supplying the P and S required by the growing mycelium.

The fact that activities of most extracellular enzymes did not vary with time does not correspond with the expectation that their production will reflect the changes in litter quality as seen *in situ* in litterbag experiments (e.g. Sinsabaugh *et al.* 2002; Šnajdr *et al.* 2011). One of the explanations might be that enzymes are constitutively produced, which is possible considering that in the wood, the natural substratum of *H. fasciculare*, the substrates of these enzymes are present in large excess. Alternatively, the enzymes might have been produced only at the beginning of the experiment before their substrates became depleted, and remain active during long periods of time, as suggested e.g. by the model of Schimel & Weintraub (2003). The ligninolytic enzymes were mostly active in the beginning of the experiment, which is in contrast with previous observations from laboratory cultures and *in situ* litterbag studies where ligninolytic enzymes were mainly detected at later stages of culture development (Hatakka 2001; Šnajdr *et al.* 2011). This might be due to the fact that they contributed to selective delignification (Mn-peroxidase) or to detoxification of low-molecular-mass phenolic compounds present in litter by their polymerization (laccase). The sharp decrease of their activity with time (and thus the relatively fast turnover) is most likely due to the fact that the oxidative enzymes are often a target of the reactive oxygen species that they produce (Hiner *et al.* 2002). It has to be noted that this study only reflected litter transformation in its initial stages with mass losses less than 40 % and that it cannot be compared with *in situ* decomposition that extends over long periods and reaches high mass losses. It is thus possible that further decomposition of litter in microcosms would also result in changes in enzyme activities.

Laccase and especially Mn-peroxidase are thought to play important roles in lignin decomposition (Hofrichter 2002; Baldrian 2006). Laccase has been reported to act in lignin decomposition, resulting in both fragmentation and re-polymerization (Leonowicz *et al.* 2001). It was demonstrated that only those litter-decomposing basidiomycetes producing Mn-peroxidase were able to substantially mineralize synthetic lignin in pure cultures (Steffen *et al.* 2000). However, in our experiment the loss of lignin was not correlated with laccase or Mn-peroxidase activity. Selective delignification was found in *Q. petraea* and *L. decidua* litters where low activities of laccase and Mn-peroxidase were recorded. In contrast, lignin removal was slow in *P. tremula* and *P. nigra* with high Mn-peroxidase activities. Similar results were reported for *Mycena inclinata* and *Marasmius quercophilus* that showed low Mn-peroxidase activities, but high lignin loss (Steffen *et al.* 2007). The fact that the ability of a ligninolytic fungus to perform selective delignification depends on litter type shows that it is difficult to draw any conclusions on the preference of the fungus for lignin/polysaccharide decomposition – a property often claimed to be inherent to certain wood-rotting or litter-decomposing fungi (Hatakka 2001; Steffen *et al.* 2007; Valášková *et al.* 2007). The present study shows that neither N nor lignin content affected the activities of ligninolytic enzymes; the cause of the negative effect of P on laccase activity remains to be clarified.

While the low lignin removal in litters with high Mn-peroxidase may be due to low production of H_2O_2 by auxiliary enzymes, high lignin removal rates in litters with low activity of ligninolytic enzymes suggests that other mechanisms of lignin decomposition, possibly based on the production of reactive oxygen radicals (Baldrian & Valášková 2008), may be active in *H. fasciculare*. One of the possible mechanisms involves the action of cellobiose dehydrogenase, an enzyme previously reported from the white-rot fungi from the genera *Irpex*, *Phanerochaete*, *Pycnoporus*, *Schizophyllum* and *Trametes* (Baldrian & Valášková 2008). Current results show that a significant decrease of Klason lignin during litter transformation can proceed also in

the virtual absence of ligninolytic enzymes (Šnajdr *et al.* 2010), which also points at the involvement of other processes in lignin transformation.

It is difficult to predict the behaviour of complex microbial communities of litter decomposers based on this simplistic study of single species decomposition. Substrata with higher N content are more suitable for bacterial growth; when litters of different N content were combined with the same initial microbial community, the relative abundance of fungi decreased with increasing N content (Hossain *et al.* 2010). N addition experiments generally tended to increase cellulose decomposition, while the activity of ligninolytic enzymes was unaffected or slightly decreased (Carreiro *et al.* 2000; Sinsabaugh *et al.* 2002; Sjoberg *et al.* 2004). It is, however, difficult to assess if this is also the case in the natural environment among the patches of litter of different N content, where N is contained in different chemical forms than after the treatments with inorganic fertilizers. The current paper also shows that the activity of ligninolytic oxidases and peroxidases probably has less effect on decomposition then expected (Sinsabaugh 2010). The observed correlations between decomposition rates and activity of these enzymes may only be a coincidence reflecting the fact that saprotrophic basidiomycetes producing these enzymes belong to efficient litter decomposers and producers of several hydrolytic enzymes. For a deeper understanding of the effects of litter chemistry on *in situ* decomposition, it would be necessary to address not only the litter transformation rates and enzyme activities but also the effects of litter chemistry on the fungal/bacterial biomass content and the fine composition of microbial communities.

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Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.funeco.2011.03.005.

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