ZENTRUM FÜR BIODIVERSITÄT UND NACHHALTIGE LANDNUTZUNG
SEKTION
BIODIVERSITÄT, ÖKOLOGIE UND NATURSCHUTZ
– CENTRE OF BIODIVERSITY AND SUSTAINABLE LAND USE –
SECTION: BIODIVERSITY, ECOLOGY AND NATURE CONSERVATION

Mycorrhizal fungi in deciduous forests of differing tree species diversity and their role for nutrient transfer

Dissertation zur Erlangung des Doktorgrades der
Mathematisch-Naturwissenschaftlichen Fakultäten der
Georg-August-Universität Göttingen
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Braunschweig

Göttingen, Juni 2014

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Tag der mündlichen Prüfung:

Für Mama.

Sieluni on sankka metsämaa.

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Summary

Mycorrhizal fungi are forming EcM on beech (*Fagus sylvatica* L.) and AM on ash (*Fraxinus excelsior* L.) and are an important component of several ecosystem processes in forests. One main functional role is nutrient acquisition, storage and subsequently transfer to the host plant in exchange for carbon from the plant.

We analyzed if several mycorrhizal fungal species in an old-growth deciduous forest in Germany differ in their functional roles for nutrient uptake and storage. For this purpose, experiments were conducted to investigate whether field-grown mycorrhizal fungi differ in their nutrient assimilation and storage of elements; and analyzed factors which might influence community composition of mycorrhizal fungi and functional abilities of mycorrhizal species.

The first experiment used plastic columns inserted 5 cm deep in the soil which were filled with seven different mixtures of ^{13}C- / ^{15}N – labelled or unlabeled leaf litter of ash and beech trees (Chapter 2 and 3).

EcM fungal species colonizing beech roots were exposed to different mixtures of ash and beech leaf litter (Chapter 2). Community composition of EcM changed after application of leaf litter and season, while number of EcM species was not affected. Hyphal biomass was higher on second harvest in October than on first harvest in May, but did not differ between leaf litter treatments.

Different EcM fungal species on root tips assimilated ^{15}N from labelled leaf litter (Chapter 3). There were species-specific differences in ^{15}N accumulation and in N concentration of mycorrhizal species and fine roots. Roots accumulated ^{15}N from labelled leaf litter. ^{15}N assimilation in mycorrhizal species increased between the two harvest dates in May and October. Whether different functional traits of EcM species might influence the observed differences for nutrient assimilation (for example nitrogen concentration or fungal exploration types) is discussed.

In the second experiment (Chapter 4 and 5), electron-dispersive X-ray microanalysis (EDX) equipped to a transmission electron microscope (TEM) was used to determine the subcellular element distribution (Mg, P, K, S, Ca) in roots of beech and ash and their associated mycorrhizas.

For this purpose, three EcM species on beech (*Clavulina cristata*, *Cenococcum geophilum* and *Lactarius subdulcis*) and one AM fungal species on ash (*Glomus* sp.) were analyzed via TEM-EDX (Chapter 4). Subcellular element concentrations were not evenly distributed throughout the cell compartments. Plant root tissues and fungal tissues differed in element concentrations. *Glomus* sp. on ash showed higher element concentration than the three EcM fungal species on beech. Differences in subcellular element concentrations were found between the three EcM fungi; *Cenococcum geophilum* showed high sulphur concentrations and *Clavulina cristata* showed high calcium concentrations than the other EcM species.

Furthermore it was tested via TEM-EDX measurements whether subcellular element concentration (Mg, P, K, S, Ca) in tree root tissues of ash and beech and fungal tissues of AM and EcM were affected by the presence of a conspecific or heterospecific neighbouring tree species (Chapter 5). Beech root tissues showed higher P and K concentrations in mixture with ash than in pure beech plots, while ash root tissues showed lower Mg, P, K, and Ca levels than in pure ash plots. AM fungal tissues showed higher element concentrations of Mg, P, K and Ca in pure ash plots, while element concentrations in EcM fungal tissues were not affected.

The findings of this study support that mycorrhizal species on beech and ash trees in an old-growth deciduous forest in Central Europe differ in their functional roles regarding nutrient uptake, transfer and storage.

List of abbreviations and acronyms

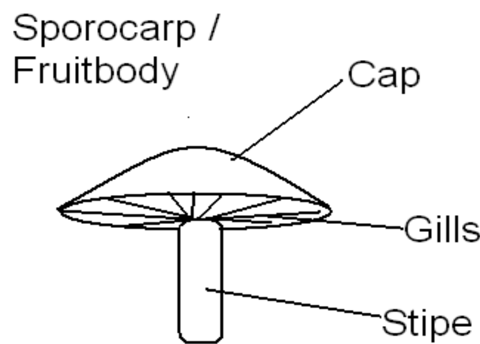
A	ash (*Fraxinus excelsior* L.)
Al	aluminium
AM	arbuscular mycorrhiza
ANOVA	analysis of variances
B	beech (*Fagus sylvatica* L.)
c	centi (10^{-2})
C	Contact Exploration Type (Agerer 2001)
C	carbon
Ca	calcium
cm	centimeter
CMM	common mycorrhizal network
^{13}C	stable carbon isotope, mass of 13 g mol $^{-1}$
°C	degree Celsius
d	day
ddH_2O	double destilled H_2O (water)
dm	decimeter
dw	dry weight
EA	elementar analyzer
EcM	ectomycorrhiza
EDX	electron dispersive X-ray analysis
ERM	extraradical mycelium
et al.	et alii
Fe	iron
g	gramm
GC	gas chromatography
h	hour
ha	hectar
K	kilo (10^{-3})
K	potassium
KOSI	Kompetenzzentrum für Stabile Isotope; Center for Stable Isotope Research and Analysis
i.e.	that means
IRMS	isotope ratio mass spectrometer
ITS	internal transcribed spacer
l	litre
LD	Long Distance Exploration Type (Agerer 2001)
m	meter (length)
m	milli (10^{-3})
M	molar
M	mega (10^{6})
µ	micro (10^{-6})
MD	Medium Distance Exploration Type (Agerer 2001)
Mg	magnesium
min	minute
mix	mixture
mono	monoculture
MT	morphotype

n	amount of substances
n	nano (10^{-9})
N	nitrogen
Na	sodium
NA	not applicable
NCBI	National Center of Biotechnology Information
NH_4^+	ammonium
NM	non-mycorrhizal
NMDS	nonmetric multidimensional scaling
NO_3^-	nitrate
n.s.	not significant
^{15}N	stable nitrogen isotope, mass of 15 g mol $^{-1}$
P	phosphorus
PAR	photosynthetically active radiation
ppm	parts per million (10^{-6})
PCA	principle component analysis
PCR	polymerase chain reaction
SD	Short Distance Exploration Type (Agerer 2001)
s	second
SE	standard error
U	units (Enzyme activity)
t	time
TEM	transmission electron microscope
UNITE	nordic ITS ectomycorrhiza database. Available at: http://unite.ut.ee/
vs.	versus

Useful terms

Fungal terminology

Fungus	A heterotrophic eukaryote, generally with stationary growth and cell walls containing chitin. Can be parasitic, saprotrophic or symbiotic.
Basidiomycete	Fungi that produce their basidia and basidiospores on or in a basidiocarp
Ascomycete	(=sac fungi). Is monophyletic and accounts for 75 % of all described fungi. Includes most of the fungi that lack morphological evidence of sexual reproduction
Sporocarp / Fruit body	The sexual structure of the fungi with sporeproducing structures.



Hyphae	Long, branching filementous structure of a fungus for vegetative growth
Mycelium	Network of hyphae
Extramatrical mycelium	Hyphae extending from the mycorrhizas into the soil, essential for nutrient uptake and transport
CMNs	Common Mycorrhizal Networks. Underground networks of hyphae created by mycorrhizal fungi that connect individual fungi and plants belowground for water and nutrient transport
Hydrophilic	= “water loving”. Hydrophilic substances are attracted to, and dissolve well in water
Hydrophobic	=“water fearing”. Substances that are repelled from water
Rhizomorph	long-distance transport hyphae, parallel-oriented, often composed of wide vessel hyphae surrounded by narrower sheathing hyphae

CHAPTER

1

GENERAL INTRODUCTION

1.1 Mycorrhiza

The term "mycorrhiza" describes a symbiosis between fungi and the roots of many terrestrial plant species. It is derived from the greek words *myko* = fungus and *rhiza* = root; meaning "fungus-root" and was first used in 1885 by Albert Bernhard Frank, who was the first one who stated that this association is beneficial for the plant (translation in Frank 2005). This mutualistic relationship is based on the exchange of nutrients provided by the heterotrophic fungus and carbon derived from photosynthesis of the autotrophic plant host. The fungi depend on the host plant for their carbon source, while they provide their hosts in return with water and nutrients, especially nitrogen and phosphorus, absorbed from the soil (Smith and Read 2008).

There are seven different types of mycorrhiza which differ in their morphological characteristics and in the fungal and plant species involved. The two main types of mycorrhiza, which occur on most plants, are the ectomycorrhiza (EcM) and the arbuscular mycorrhiza (AM). Other mycorrhizal types are the orchid mycorrhiza, ericoid mycorrhiza, arbutoid mycorrhiza, monotropoid and the ectendomycorrhiza (Peterson and Massicotte 2004; Smith and Read 2008).

1.2 Ectomycorrhiza (EcM)

Ectomycorrhizal symbiosis is formed between many soil fungal species (with approximately 7000 to 10.000 fungal species, which are mainly Basidio- and Ascomycetes; Lilleskov et al. 2011) and the roots of their plant hosts (approximately 8000 host species; Taylor and Alexander 2005). This symbiosis is mainly found in temperate and boreal ecosystems, but also appears on some tropical plant species. EcM symbiosis evolved between 220 and 150 million years ago (Selosse and LeTacon 1998, Bruns and Shefferson 2004).

The two main structures of ectomycorrhizas are the hyphal mantle and the Hartig Net. The fungus forms a sheath of hyphae around the plant root (Fig. 1). The fungus also penetrates with hyphae intercellularly between the rhizodermis and cortex of the plant root, and forms the so-called Hartig net (Fig. 1), which is named after Robert Hartig, a mycologist who first observed these structures.

These characteristics and function of these structures of EcM may differ between fungal species (Kottke and Oberwinkler 1986). The hyphal sheath prevents direct contact between the root tip and the soil matrix and is hydrophobic in many cases, but can also have hydrophilic properties (see below; Agerer 1987-2008; Agerer 2001). The Hartig Net is a network of specialized cells, forming an interface where the nutrient exchange between fungus and host takes place.

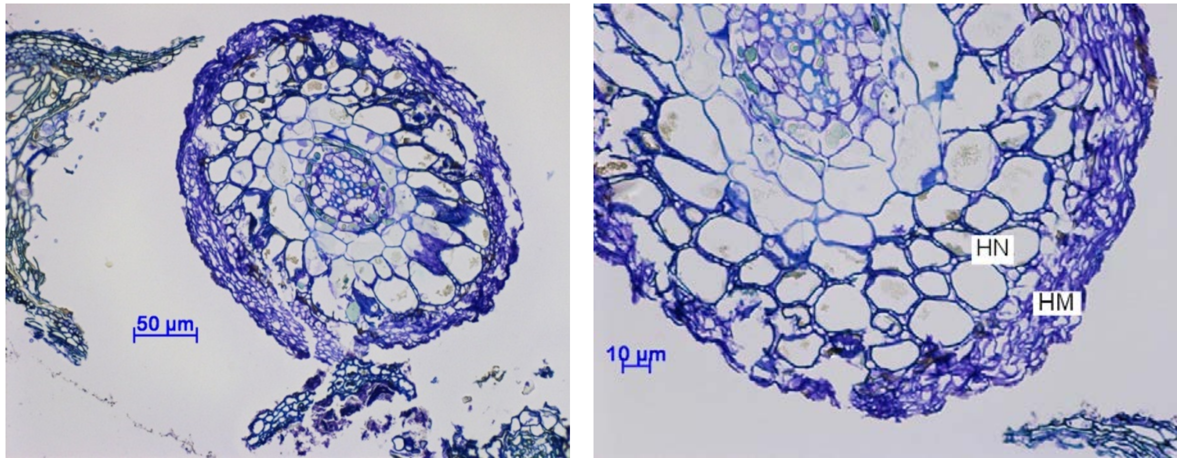


Fig. 1: Light microscopy of roots of beech (*Fagus sylvatica* L.) colonized by an ectomycorrhizal (EcM) fungal species (*Lactarius subdulcis*). HM = hyphal mantle, HN = Hartig net.

Nutrients and water are taken up via the extraradical mycelium (ERM) and transported to the host plant. Compared to non-mycorrhizal plants, the hyphal mantle as well as the ERM increases the nutrient absorbing surface of the root tip; therefore, the access to nutrients is increased (Finlay 2008).

Aboveground structures are fruitbodies of the fungi, which serve as reproductive organs. Some of the most widely known fungi in the forest like the fly agaric (*Amanita muscaria*) are ectomycorrhizal fungi.

There are several tools to identify mycorrhizal fungi. The most widely used technique is a combination of morphotyping and molecular DNA methods aiming a quantitative as well as qualitative analysis of the mycorrhizal community. Ectomycorrhizal fungal root tips exhibit typical "morphotypes" according to their anatomical and morphological features, like mantle structure, branching, colour and presence of rhizomorphs, which can be used for their distinctions. Agerer (2001) developed the concept of exploration types of EcM fungi according to their abundance and length of hyphae, mantle anatomy and production of rhizomorphs. This classification is ecologically important because fungi of different exploration types are able to exploit nutrients from distant patches. EcM fungal mantles can be either hydrophobic due to water-repelling substances or hydrophilic. Fungi of the short distance (SD) exploration type are characterized by short, but dense emanating hyphae without rhizomorphs. The contact (C) exploration type shows a smooth mantle and no or only very few emanating hyphae. Rhizomorphs are absent. The medium distance (MD) exploration type is separarted into smooth and fringe subtypes, may have rhizomorphs and is able to transport water and nutrients over medium distances from the soil to the root hyphae (less than 1 cm). The long distance (LD) exploration type has few but highly differentiated rhizomorphs to transport nutrients over long distances, up to several dm from the root surface.

1.3 Arbuscular Mycorrhiza (AM)

The arbuscular mycorrhiza (AM) is an obligate, the most ancient and widespread form of mycorrhizal symbiosis. A wide range of plant species (as many as 250.000) like grasses, shrubs, tropical trees and some trees in temperate regions form this symbiosis, together with only approximately 200 descibed AM fungal species (Schüßler and Walker 2010; Redecker et al. 2013). Redecker et al. (2000) estimated from fossilized fungal structures that the first land plants formed with Glomalean fungi about 460 million years ago.

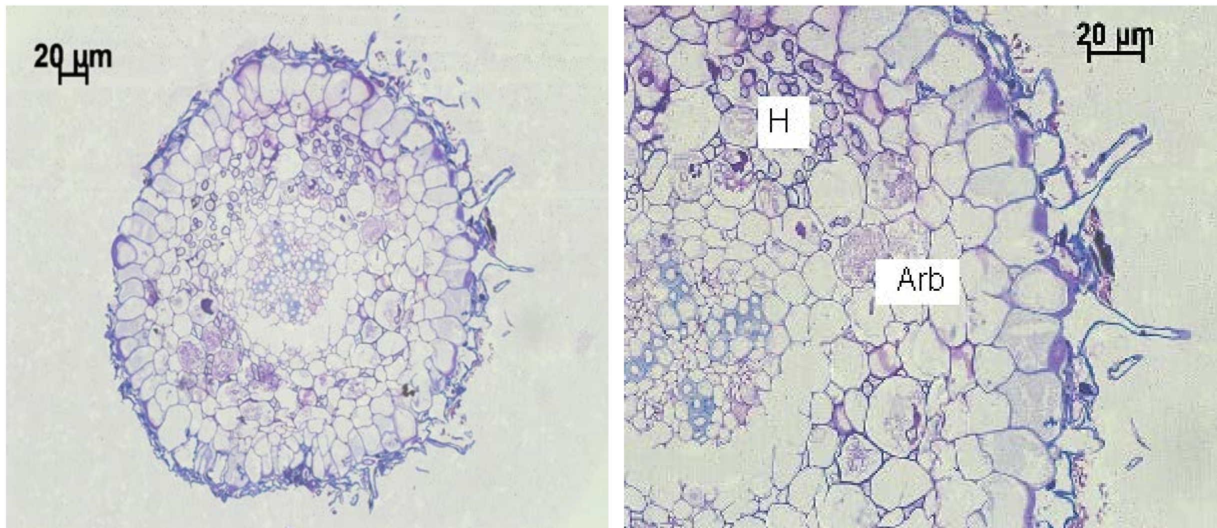


Fig. 2: Light microscopy of ash tree roots (*Fraxinus excelsior* L.) colonized by an arbuscular mycorrhizal (AM) species (*Glomus* spec.) H = hyphae, Arb = arbuscles.

Typical structures of this type of mycorrhiza are the name-giving arbuscles (Fig. 2), which are branched tree-like structures inside the cortex cells of the host plant, where the nutrient transfer from the fungus to the plant (and vice versa) takes place. Very important in this context is the interfacial membrane which surrounds the arbuscle, its surface is enhanced through the branching of the small arms of the arbuscles. This interfacial membrane grows intercellularily, without penetrating the host plasmalemma (Peterson and Massicotte 2004).

Further important structures of AM symbiosis are vesicles, small round structures containing lipids and serving as storage organs. These structures are connected by inter- and intracellular hyphae (Fig.2) which also emanate from the root into the surrounding soil, where the absorption and uptake of nutrients takes place (Leake et al. 2004).

Identification and visualization of AM is more difficult than identification of EcM because these fungal structure grow inside the roots. For AM detection, the root must be bleached and acidified. Thereafter, the fungal structures can be stained with different fungal specific dyes.

1.4 Mycorrhizal associations on trees

Each biome is dominated by a certain type of mycorrhiza (Read 1991). In temperate forests in Central Europe, the majority of forest trees (*Fagus*, *Pinus*, *Picea*, *Abies*, *Tilia*, *Betula*) is associated with the ectomycorrhizal symbiosis (EcM). Other tree species usually form the arbuscular mycorrhizal (AM) symbiosis, like the genera *Fraxinus*, *Acer* and *Aesculus*. For some other tree species (*Populus*, *Alnus*, *Quercus*, *Eucalyptus*, *Salix*), it is widely recognized and known that these species do inhabit AM as well as EcM, often simultaneously or in different developmental stages (Harley and Harley 1987; De Roman 2005).

1.5 Taxanomic diversity, community structure and host preferences of mycorrhizal fungi

Because 90 to 99 percent of all living root tips in a forests are colonized by mycorrhizal fungi, the mycorrhizal symbiosis has been suggested to be a very important component of the forest ecosystem. Mycorrhizal fungi show a great taxanomic diversity in boreal and temperate forests (Horton and Bruns 2001; Bruns 1995; Tedersoo et al. 2010). In many cases, an average number of 50 to 100 fungal species per forest stand has reported (Horton and Bruns 2001; Buée et al. 2005; Dickie 2007; Twieg et al. 2009; Lang et al. 2011).

Molecular biology offers reliable tools for identification of species (Horton and Bruns 2001). After visual examination of mycorrhizal species, DNA can be isolated from mycorrhizal root tips followed by PCR-based analysis of the nuclear ribosomal internal transcribed spacer (ITS) region (Horton and Bruns 2001; Anderson and Cairney 2004). For AM fungi, a “nested” PCR can be used, where specific AM primers are combined with ITS primers (Renker et al. 2003). New sequencing tools like 454 pyrosequencing offer a further opportunity to screen the soil community and estimate the microbial diversity in the soil with high throughput-rates of samples (Buée et al. 2009a; Öpik et al. 2009).

There is high spatial and temporal dynamics within the mycorrhizal community and several biotic as well as abiotic factors influence the community structure of mycorrhizal fungi and occurance of certain mycorrhizal fungal species (Taylor et al. 2002; Tedersoo et al. 2003; Buée et al. 2005; Toljander et al. 2006; Jarvis et al. 2013). The most important ones are soil parameters (nutrient availability, nitrogen content, pH, soil moisture), preferences or specificity for host genotype and age, sampling time and sampling volume, disturbances like fire and windfall (succession), heavy metal contamination, CO_2 concentration, interactions with other soil organisms and chronosequences (reviewed in Chai et al. 2013).

For example, increased mineral N availability in soil can lead to changes in the EcM fungal community structure and reduced species richness (Wallenda and Kottke 1998; Lilleskov et al. 2011). Some EcM species like *Cortinarius, Piloderma* and *Suillus* are sensitive to enhanced levels of soil N (Taylor et al. 2000; Lilleskov et al. 2002), while other EcM species like *Laccaria, Lactarius, Paxillus* and *Russula* have been found to increase fruit body production with increased levels of soil N (Lilleskov et al. 2001; Avis et al. 2003). In addition, the amount of external mycelium of EcM fungi has been found to decrease significantly after N additions in both laboratory studies (Wallander and Nylund 1992; Arnebrandt 1994) and in the field (Nilsson and Wallander 2003). But the response is context dependant because Kåren and Nylund (1997) found no reduced species richness after mineral N addition.

Tree species diversity and host preferences of mycorrhizal fungi influence mycorrhizal fungal communities (Ishida et al. 2007; Tedersoo et al. 2008; Lang et al. 2011). There is accumulating evidence that also tree species identity shapes the mycorrhizal community and abundance of certain mycorrhizal species (Haskings and Gehring 2004; Johnson et al. 2005; Hubert and Gehring 2008; Morris et al. 2009; Kohout et al. 2011; Prescott and Grayston 2013). Tree species indirectly influence the microbial community in the soil via their leaf litter fall. Leaf litter application or removal of leaf litter leads to changes in diversity or community composition of EcM fungi (Conn and Dighton 2000; Cullings et al. 2003; Smit et al. 2003; Aponte et al. 2010). However, the extend in which certain EcM species are affected by changes in leaf litter application is not fully known.

The differences in responses to variation in environmental conditions such as changed levels of N, leaf litter, tree species diversity or identity may, to some degree, reflect functional differences between EcM fungal species or genera.

1.6 The functional diversity of mycorrhizal fungi in the nutrient cycling of forests

Besides the high taxanomic diversity, mycorrhizal fungi also display multifunctional roles in different ecosystem processes (Newton 1992; Cairney 1999; Allen et al. 2003; Finlay 2004; 2008; Buée et al. 2005; McGuire et al. 2010). EcM fungal species differ in their ability to exploit soil nutrients, which also might be an explaination for the uneven distribution of EcM fungal species in a forest stand (Cullings and Courty 2009, Courty et al. 2010a, Diedhou et al. 2010).

In nature, most nutrients are bound in complex organic macromolecules, but trees are not able to take up nutrients in an complex organic forms. Therefore fungi degrade these compounds and supply them to the plant. The extraradical mycelium (ERM) of the mycorrhizal fungi accesses these nutrients over far distances in the forest soil (Finlay 2008). The benefit for plants is an increased uptake of nutrients not easily accessable for plants. This leads to improved growth and development of mycorrhizal plants compared to non-mycorrhizal ones, especially under nutrient-limiting conditions.

Mycorrhizal fungi are able to utilize organic nitrogen and phosphorus from complex substrates like leaf litter and humus, and some mycorrhizal species have been shown to differ in this abilities in laboratory experiments (Perez-Moreno and Read 2000; Read and Perez-Moreno 2003; Smith and Read 2008). If mycorrhizal species in the field differ in their functional roles for nutrient assimilation is poorly understood (Finlay 2004; Pena et al. 2013).

Fungi transfer nutrients to their host plants, which is regulated by host plant demand. If fungi store the accumulated nutrients in their own tissues, this is also important since differences in nutrient status of the fungus may also influence their decomposition (Koide and Malcolm 2009) and their use as food source. Mycorrhizal species have shown to differ in their nutrient accumulation and storage in the laboratory (Kottke et al. 1998) and in the field (Rumberger et al. 2005); but the extend in which several mycorrhizal species differ in nutrient storage and element distribution is yet unclear.

1.7 Nitrogen cycling in EcM mycorrhizal symbiosis

Mycorrhizal fungi are important for N uptake and storage and lead to an improved uptake of ammonium and organic N (Chalot et al. 2002; Plassard et al. 2000), and to a lesser extend of nitrate (Plassard et al. 2000; Nygren et al. 2008). In forests (low pH and low nitrification rates), N is taken up preferentially as ammonium by mycorrhizal fungi (Kottke and Oberwinkler 1986). EcM fungi are able to produce the major enzymes necessary for the degradation of organic N (Chalot and Brun 1998; Courty et al. 2010a), because EcM symbiosis has evolved from saprotrophic ancestors (Hibbett et al. 2000). Production of extracellular proteases in EcM fungi and growth on protein-substrates has been demonstrated (Read and Perez-Moreno 2003). Abuzinadah and Read (1986) made an attempt to categorise EcM fungi as protein or non-protein fungi, depending on the abilities of the fungi to use protein sources. But EcM fungi also show intraspecific variation (Cairney 1999). Leaf litter is one important source of N in forests. ^{15}N experiments show a rapid incorporation of available N into EcM fungal biomass under field conditions and it was demonstrated that ^{15}N from labelled beech litter can be detected after 6 months in roots and beech leaves, suggesting that ectomycorrhizal fungi mainly use soil organic N (Zeller et al. 2000). ^{15}N stable isotopes are an important tool to investigate fungal functioning in natural ecosystems and functional differences between mycorrhizal species (Hobbie et al. 2014). Pena et al. (2013) showed interspecific differences in N acquisition of EcM from labelled leaf litter. Whether different EcM species in the mineral horizon of a forest differ in their N assimilation from leaf litter is yet unclear.

1.8 Phosphorus cycling and elmental composition in EcM and AM mycorrhizal symbiosis

Phosphorus (P) is an essential nutrient element necessary for growth and development of plants. It is incorporated in DNA, nucleid acids and amino acids. Although the total amount of P in soil is high, it is a very immobile and inavailable element for plant uptake. The concentration of inorganic P (Pi) in the soil is low (<10 mM; Marschner 1995; Hinsinger 2001).

Mycorrhizal fungi and especially their extraradical mycelium (ERM) play an important role in acquisition of P from the soil solution (Bolan 1991; Smith and Read 2008; Cairney 2011). Different EcM fungal species differ in their ability to mobilise and take up P from the soil, and also for AM the possibly involved phosphate transporters have been identified (Plassard et al. 2011 and reference therin). Perez-Moreno and Read (2000) showed that colonization of leaf litter by Paxillus involutus led to a loss of P from the leaf litter after 90 days. Flux of P in roots colonized by mycorrhizal fungi can be 3- to 5 times higher than in NM roots and differences between different EcM species were found, with *Paxillus involutus* being the more effective for Pi uptake than *Thelephora terrestris* and two *Suillus* species (Colpaert et al. 1999; Van Tichelen and Colpaert 2000).

Other mineral nutrients (Mg, Ca, K, S) are translocated in mycorrhizal mycelia and stored in fungal tissues, but were seldom studied since these nutrients are usually not limited in natural ecosystems. Mycorrhizal fungi translocate K and Mg (Jentschke et al. 2001), and are able to mobilize potassium from apatite (Wallander 2000). Nutrient uptake has been analyzed with radioactive tracers (Kuhn et al. 2000), but to date comparably little is known about differences between mycorrhizal species regarding nutrient uptake and storage of these elements (Smith and Read 2008). Whether different EcM and AM fungal species in the forest differ in their element concentration and whether these elements concentrations are affected by heterospecific neighbour trees is not fully known (Rumberger 2004).

1.9 Study area

The study area was in the Hainich national park, one of the largest old-growth deciduous forests of Europe located in Thuringia, Germany (7500 ha). The forest is dominated by the European beech (*Fagus sylvatica* L., >95% of total basal area), but because of former management practices, there are up to 14 different tree species present in some parts of the Hainich (e.g. *Fraxinus excelsior*, *Carpinus betulus, Tilia* sp., *Acer* sp., *Prunus avium*, *Quercus robur*, *Quercus petraea*, *Ulmus glabra*).

Several research sites (study plots of 50x50 m) located in the north east of the national park were installed in the forest, and have been characterized in former studies (e.g. Mölder et al. 2006; 2008; Leuschner et al. 2009; Guckland et al. 2009; Jacob et al. 2009; Talkner et al. 2009).

In this study beech (*Fagus sylvatica* L.) and ash (*Fraxinus excelsior* L.) trees were used because they associate with different mycorrhizal types (EcM on beech trees and AM on ash trees). These tree species differ in the chemical composition of their leaf litter (Jacob et al. 2009). Presence of ash trees and application of ash leaf litter in a beech forest influences soil properties and nutrient status (Guckland et al. 2009; Talkner et al. 2009; Langenbruch et al. 2012) and could possibly influence belowground processes and the soil microbial community (e.g. Cesarz et al. 2013). If the presence of ash trees in a beech forest has implications for the mycorrhizal community, especially for the main mycorrhizal species, is not known.

1.10 Objectives of the study

The study was conducted within the Research Group ("Graduiertenkolleg") 1086 "The role of biodiversity for biogeochemical cycles and biotic interactions in temperate deciduous forest" funded by the DFG (Deutsche Forschungsgemeinschaft; German Science Foundation) in a natural deciduous forest, the Hainich National park in Thuringia, Germany.

The overall aim of this work was to investigate the functional diversity of field-grown mycorrhizal fungal species for nutrient transfer and to determine factors which influence mycorrhizal communities and lead to changes in functional traits of mycorrhizal fungal species.

The specific objectives were:

- To determine the influence of pure and mixed leaf litter of ash and beech trees on the ectomycorrhizal community composition and growth of hyphae in a pure beech stand. **(Chapter II).**
- To examine if ectomycorrhizal fungi in a pure beech stand contribute to and differ between species in nitrogen uptake from differently labelled leaf litter of beech and ash. The flow from ^{15}N labelled leaf litter to mycorrhizal root tips and roots was examined. In this context, it was analyzed if EcM species and root differ in N concentration (**Chapter III).**
- To investigate differences in the subcellular element localization (phosphorus, calcium, potassium, sulphur and magnesium) and storage of different ectomycorrhizal species on beech and on an arbuscular mycorrhizal species on ash. **(Chapter IV).**
- To examine if the presence of a heterospecific neighbour tree influences the subcellular element contents in roots of beech and ash and their associated mycorrhizas (**Chapter V).**

CHAPTER

2

Leaf litter composition affects subjacent ectomycorrhizal assemblages but not hyphal abundance

2.1 Abstract

The present study aimed to investigate the effect of plant leaf litter species and leaf litter decomposition on the colonizing ectomycorrhizal fungal morphotypes on beech roots in the field.

A leaf litter decomposition experiment for one year was conducted, with seven different litter mixtures of beech and ash leaf litter as treatments. Root and mycorrhizal parameters as species composition of EcM fungi, number of EcM species and hyphal biomass were analyzed.

There were seasonal and treatment-related effects on mycorrhizal community structure and seasonal effects on extracted hyphal biomass. EcM fungal species on root tips differed in abundances in leaf litter treatments and harvest dates. There was a separation of the EcM community between harvest dates and between pure and mixed leaf litter treatments on the second harvest in october. This led to changes in dominance of certain exploration types of fungi. Hyphal biomass was higher in October compared to May, but was not significantly different between leaf litter species.

Application of ash leaf litter and mixtures of leaf litter influence abundance of EcM fungi in a pure beech forest stand. Tree leaf litter and correlating parameters like decomposition rate, N release and moisture have an influence on EcM fungal community structure on root tips. Hyphal biomass is determined by season.

2.2 Introduction

Leaf litter fall and its decomposition are important processes in the nutrient cycle of temperate broadleaved forests. Because plant leaf litter consists of labile (sugars, cellulose, amino acids) as well as recalcitrant (lignin) substrates, the leaf litter decomposition rate varies between tree leaf litter species (Jacob et al. 2010). It is regulated by several factors, e.g. temperature, moisture and pH value, nutrient concentrations and ratios and the lignin content of the leaf litter. Beech (*Fagus sylvatica* L.) litter and ash (*Fraxinus excelsior* L.) litter are completely different in this sense (Jacob et al. 2010). Also, release of nutrients from these substrates differs.

Ectomycorrhizal (EcM) fungi play an important role in the nutrient uptake in forests and transfer of these nutrients to their host plants; one of the main nutrient sources is decaying litter (Bending and Read 1995; Perez-Moreno and Read 2000). EcM fungi can mainly be found in the surface litter layer, where the mycelium forms the connection between the litter substrate through the soil and the mycorrhizal root tips, between several root tips and even between trees, forming an intimate connection for nutrient transfer processes in a common mycorrhizal network (CMN; Finlay 2008).

EcM fungi are taxonomical and functional diverse. Number of species and community composition of EcM fungi changes due to different abiotic as well as biotic factors. Nutrient availability is one of the major determining factors. Application of different litter substrates to the soil surface or even removal of the leaf litter layer leads to changes in EcM community and EcM species richness. Conn and Dighton (2000) found that application of different oak litter and pine needle substrates leads to changes in EcM community. Brearley et al. (2003) reported lower EcM diversity in a tropical nursery and Cullings et al. (2003) found a decrease in EcM species richness in a pine forest after litter addition. Removal of litter and humus led to an increase in the number of species and fruiting bodies of EcM fungi (Smit et al. 2003). Aponte et al. (2010) concluded that oak host species indirectly affect EcM fungal communities through leaf litter fall and quality of leaf litter.

However, the effects of leaf litter on mycorrhizal fungal species on root tips under natural conditions in a forest ecosystem are still poorly understood; and the extend in which selected EcM fungal species differ in their behaviour after leaf litter application is yet unclear. Most studies focused either on deciduous trees in combination with coniferous trees or tropical tree species.

The aim of this study was to determine the influence of application of leaf litter species mixtures containing ash (an arbuscular mycorrhiza forming deciduous tree species) leaf litter on EcM fungi in a pure beech stand, where only EcM fungal species occur. To differentiate between tree species effects and leaf litter quality effects we produced leaf litter of beech and ash in the greenhouse, altering N content and decomposition rate of the leaf litter treatments. We hypothesize that EcM

fungi community composition and number of EcM fungal species changes as a result of a different leaf litter treatment (mixtures of beech and ash leaf litter, or even pure ash or beech leaf litter). We also hypothesize that hyphal length in soil changes as a result of a different leaf litter treatment.

2.3 Materials and methods

2.3.1 Production of plant litter for the field experiment

One-year old beech (*Fagus sylvatica* L.) and ash (*Fraxinus excelsior* L.) plants were planted into Hainich soil in 100-l-boxes in a greenhouse for one growing season (09.04.2008 to 14.10.2008) under the following conditions: temperature 22.8 ± 2.8 °C, and air humidity of 71.8 ±13 %. The plants were grown with supplementary light under long-day conditions (16 h light, 8 h dark) achieving 85 ± 30 µmol $m^{-2}s^{-1}$ of photosynthetic active radiation (PAR) at plant height. The plants were supplied with a Hoagland–based nutrient solution (0.6 mM $CaCl_2*2H_2O$, 0.04 mM $MgSO_4*7H_2O$, 0.01 mM $FeCl_3*6H_2O$, 2 mM NH_4NO_3, 0.4 mM KH_2PO_4, 1.8 µM $MnSO_4*H_2O$, 0.064 µM $CuCl_2*2H_2O$, 0.15 µM $ZnCl_2$, 0.1 µM MoO_3, 0.01 mM H_3BO_3. The soil moisture was kept at 30 %.

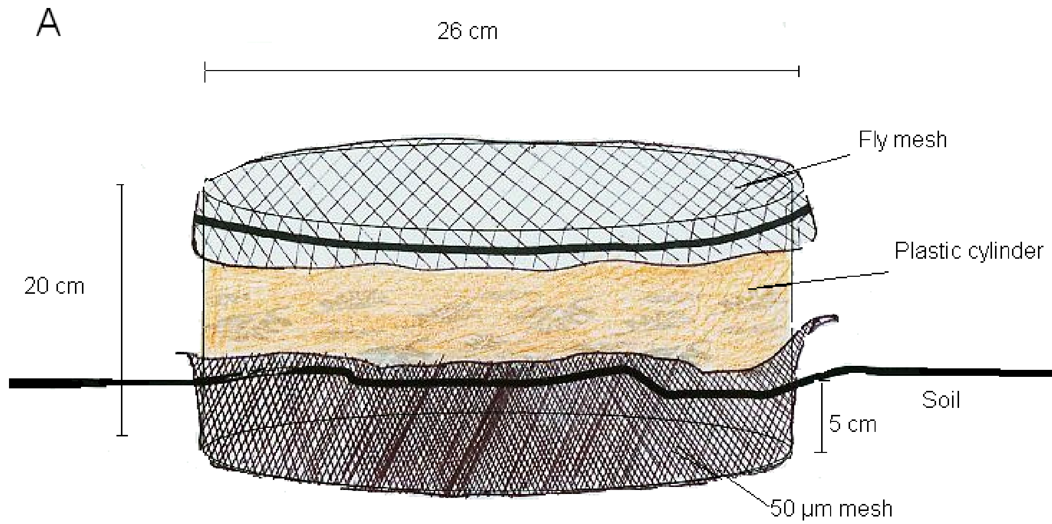
At the end of the growing season, leaves were removed from the plants, air dried and stored in paper bags at room temperature. Natural leaf litter of ash and beech was collected in the Hainich forest in autumn 2008, air dried and stored in paper bags at room temperature.

2.3.2 Setup of the Decomposition experiment

The experimental plot (50x50m) in a monospecific 100 to 200-year-old beech stand located in Thuringia, Germany (51°05'28''N, 10°31'24''E) was fenced. The forest was unmanaged in the last four decades. The long-term mean of annual precipitation is 670 mm and the annual mean temperature is 7.5 °C (Leuschner et al. 2009). The geological substrate is limestone covered by loess (Guckland et al. 2009). The soil from the site is classified as luvisol. The pH (H_2O) of the soil in the experimental plot was 4.2 to 4.4 (Guckland et al. 2009).

The experimental treatments started on 9th of December 2008 using cylinders of a diameter of 26 cm and a height of 20 cm placed in the forest soil in the field (Fig. 1). A soil corer with the same diameter as the cylinders was used to place an undisturbed core of forest soil into the cylinder to a depth of 5 cm without disturbing the soil horizons. Small seedlings were cut off, if present. To prevent roots to grow into the cylinder, a nylon mesh (mesh size 50 µm) was fixed under the bottom; therefore, only ingrowth of hyphae was possible. Inside the soil-filled cylinders, the litter on the soil surface was removed and replaced with 14.38 g of litter per cylinder.

Figure 1: Experimental setup. Scheme of a cylinder containing an undisturbed soil core (A). A nylon mesh was fixed under the bottom to prevent root ingrowth (B). Cylinders were placed in a beech forest and covered with a fly mesh to avoid additional leaf litter input (C). Before placement in the soil, the surface litter was removed and replaced by 14.38 g of litter mixtures as described in Table 1.



We installed four subplots each containing 4 replicates of 7 litter treatments. The litter treatments were either beech or ash litter from the greenhouse (high nitrogen) or the forest (low nitrogen) and combinations of equal amounts of ash and beech litter from forest or greenhouse (intermediate nitrogen) (Table 1). There were 8 replicates of each litter treatment. This design resulted in a total of 56 cylinders per harvest date (2 harvest dates). Within each of the four subplots, the cylinders with the seven different litter treatments were randomly distributed. To prevent loss of litter by external factors (wind, animals) the cyclinders were covered with fly mesh (mesh size 1 mm).

Table 1: Leaf litter treatments used in the litter decomposition experiment. N content indicates the amount of N present in the litter (mg per cylinder) applied at the start of the experiment on the forest soil. Different letters indicate significant differences at p<=0.05. Asterisks (*) indicate greenhouse leaf litter types (plants grown in the greenhouse). $Greenhouse_A$=Ash leaf litter grown in the greenhouse, $Greenhouse_B$ = Beech leaf litter grown in the greenhouse, $Forest_A$ = Ash leaf litter collected in the forest, $Forest_B$ = Beech leaf litter collected in the forest. Data were kindly provided by C. Langenbruch.

Treatment	Abbreviation	Growth condition	N content (mg)
Beech	B	Forest	125
Beech	B*	Greenhouse	305
Ash	A	Forest	164
Ash	A*	Greenhouse	287
Beech+Ash	BA	Forest	144
Beech+Ash	B*A	$Greenhouse_B$+$Forest_A$	235
Beech+Ash	BA*	$Forest_B$+$Greenhouse_A$	206

2.3.4 Harvest and sample collection

Harvests took place 5 months (May 5th 2009) and 11 months (October 20th 2009) after the start of the experiment in December 2008. One soil core (diameter 5 cm, depth 5 cm) including surface litter was taken from each cylinder for hyphal extraction. Roots were not present in the soil inside the cylinders. Samples underneath the cylinders were used for determination of the ectomycorrhizal communities on root tips. For this purpose, six randomly positioned soil cores (diameter 5 cm, depth 5 cm, each of a volume of about 100 cm^3) were taken below the cylinder and then mixed to result in one sample per cylinder. All samples were stored at 4 °C until further analysis.

The surface litter of each cylinder was removed, air-dried and weighed. The C and N concentrations of the litter at the beginning and at each harvest were determined in an elemental analyzer (Heraeus Elementar Vario EL, Hanau, Germany) (Langenbruch et al. 2014). Litter decomposition was determined as: (litter mass at harvest * 100) / litter mass at start.

2.3.5 Hyphal length

For hyphal extraction, 5 g of soil were added to 50 ml deionized water, carefully shaken, mixed and stored overnight in a flask (Hanssen et al. 1974). After sedimentation of soil particles, the hyphae were floating on the surface. Hyphae and water were pipetted with a glass pipette to a cellulose nitrate filter (pore size 1.2 µm; Sartorius Stedim Biotech GmbH, Göttingen, Germany). Hyphae were retained by the filter, stained with trypan blue (0.05 % trypan blue in 50 ml glycerine, 45 ml ddH_20, 5 ml HCl) and observed under a binocular (Leica Microsystems, Wetzlar, Germany) and photographed. Length of hyphae was determined using Image J (http://rsbweb.nih.gov/ij/).

2.3.6 Analysis of roots and mycorrhizal fungi

The soil samples collected below each cylinder were used for mycorrhizal analysis. For this purpose the roots were carefully washed to remove soil particles and then used to count the abundance of living non-mycorrhizal, mycorrhizal and dead root tips under a binocular (Stemi SV 11, Zeiss, Jena, Germany). The mycorrhizal root tips were classified according to morphotypes (MT) under the microscope (Stemi SV 11, Zeiss, Jena, Germany) applying morphological and anatomical characteristics (Lang et al. 2011 and http://www.uni-goettingen.de/de/92389.html). After morphotyping, fine roots were dried and weighed, and morphotypes were frozen at -80°C for molecular analysis.

For the molecular identification of the fungi, the MTs were ground in a mill (Type MM2, Retsch, Haan, Germany) and subjected to DNA extraction with the DNAeasy Mini Plant Kit (Quiagen, Hilden, Germany). ITS sequencing using the fungal specific primer pair ITS1F and ITS4, cloning and Gene Bank and UNITE comparisons were done according to Lang et al. (2011). The sequences have been deposited in NCBI GenBank with the GenBank accession numbers: KC952674-KC952675, KC952677-KC952681, KC952686, KC952688, KC952691, KC952692, KC952704, KC952707 and KC952710. Species identification and abundance data are shown in supplement Table S1.

2.3.7 Statistical analysis

Eight cylinders per litter treatment were harvested for mycorrhizal observation and analysis. Where appropriate, data are indicated as means ± SE. Calculation of diversity indices was conducted in PAST (Hammer et al. 2001). Statistical analysis was conducted in R (R Development Core Team (2009); http://www.r-project.org). Nonmetric Multidimensional Scaling (NMDS) and Priciple Component Analysis (PCA) were conducted in R using the Vegan package (Oksanen et al. 2005).

ANOVA followed by Tukey's HSD Test was used to detect differences in EcM fungal abundance. When the criteria of normal distribution and homogeneity of variance were not satisfied, differences between medians were compared by non-parametric multiple comparisons (Mann-Whitney test). Treatment effects were considered significant, if $P \leq 0.05$ and were indicated using different letters.

2.4 Results

2.4.1 Diversity of mycorrhizal assemblages under ash, beech and mixed leaf litter

We counted a total of 9449 mycorrhizal root tips and identified 15 EcM species under different leaf litter combinations of ash and beech in May and 17296 mycorrhizal root tips and 14 EcM species in October. Root mass did not differ between the sampling dates (Table 2). The total EcM species richness was 17. Samples for each treatment were analyzed to EcM species saturation (Fig. 2 A,B). The most abundant EcM species in this study were *Cenococcum geophilum, Lactarius subdulcis*, *Thelephora* spec., *Clavulina* spec. (May) and *Xerocomus* spec. (October). There were no differences in EcM species richness, EcM colonization, vitality, or Eveness between the sampling dates or between samples exposed to ash, beech or mixed litter treatments (Table 2). The diversity indices (Shannon, Simpson, Berger-Parker, Fischer´s α) of the EcM assemblages did not differ between May and October or between EcM assemblages exposed to different litter treatments (Table 2).

Table 2: Root mass, mycorrhizal colonization, vitality, and diversity indices of ectomycorrhizal communities under beech, ash or mixed beech-ash litter. N = 8. Asterisks (*) indicate greenhouse leaf litter types (plants grown in the greenhouse). A= Ash leaf litter, B = Beech leaf litter, BA = mixture of ash and beech leaf litter.

Parameter	Season	Treatment	Root Mass [g/0.6 l sample]	MC [%]	VI [%]	Taxa[S]	Taxa by Chao 1	Simpson (1-D)	Shannon H´	Evenness	Fisher α	Berger-Parker
B	May	B	2.07 ±0.38	100.00 ±0.00	43.00 ±5.58	10	10	0.79	1.75	0.58	2.78	0.30
B*	May	B	1.73 ±0.21	99.02 ±0.51	32.35 ±4.48	10	10	0.80	1.87	0.65	2.78	0.32
A	May	A	2.14 ±0.49	97.62 ±0.89	35.66 ±5.83	12	12	0.81	1.94	0.58	3.47	0.33
A*	May	A	1.97 ±0.40	96.26 ±1.56	33.64 ±4.42	9	9	0.80	1.85	0.71	2.40	0.34
BA	May	M	2.08 ±0.43	97.99 ±0.81	29.29 ±3.60	9	9	0.83	1.93	0.77	2.40	0.24
B*A	May	M	2.16 ±0.65	98.21 ±1.06	28.81 ±4.34	10	10	0.79	1.80	0.61	2.77	0.33
BA*	May	M	2.31 ±0.37	99.14 ±0.51	36.90 ±4.70	9	9	0.81	1.83	0.69	2.40	0.31
B	October	B	1.98 ±0.30	74.94 ±8.19	43.21 ±7.59	11	11	0.80	1.87	0.59	3.15	0.33
B*	October	B	2.76 ±0.31	97.98 ±0.94	34.37 ±4.49	7	7	0.71	1.46	0.617	1.73	0.46
A	October	A	2.39 ±0.41	99.26 ±0.45	39.09 ±3.26	9	9	0.80	1.83	0.692	2.40	0.33
A*	October	A	3.08 ±0.36	95.54 ±4.08	34.22 ±3.85	10	10	0.83	2.01	0.747	2.77	0.31
BA	October	M	1.84 ±0.36	97.71 ±1.03	37.47 ±4.39	9	9	0.81	1.86	0.712	2.40	0.31
B*A	October	M	1.75 ±0.35	98.00 ±1.06	41.37 ±4.92	11	11	0.79	1.78	0.540	3.15	0.30
BA*	October	M	2.68 ±0.50	98.33 ±0.59	41.32 ±3.80	10	10	0.79	1.75	0.577	2.77	0.31
P (date)			0.18	0.93	0.08	0.58	0.58	0.35	0.41	0.92	0.61	0.29
P (treatment)			0.67	0.50	0.34	0.88	0.88	0.23	0.23	0.41	0.91	0.16
I (date x treatment)			0.37	0.19	0.49	0.57	0.57	0.41	0.65	0.31	0.57	0.23

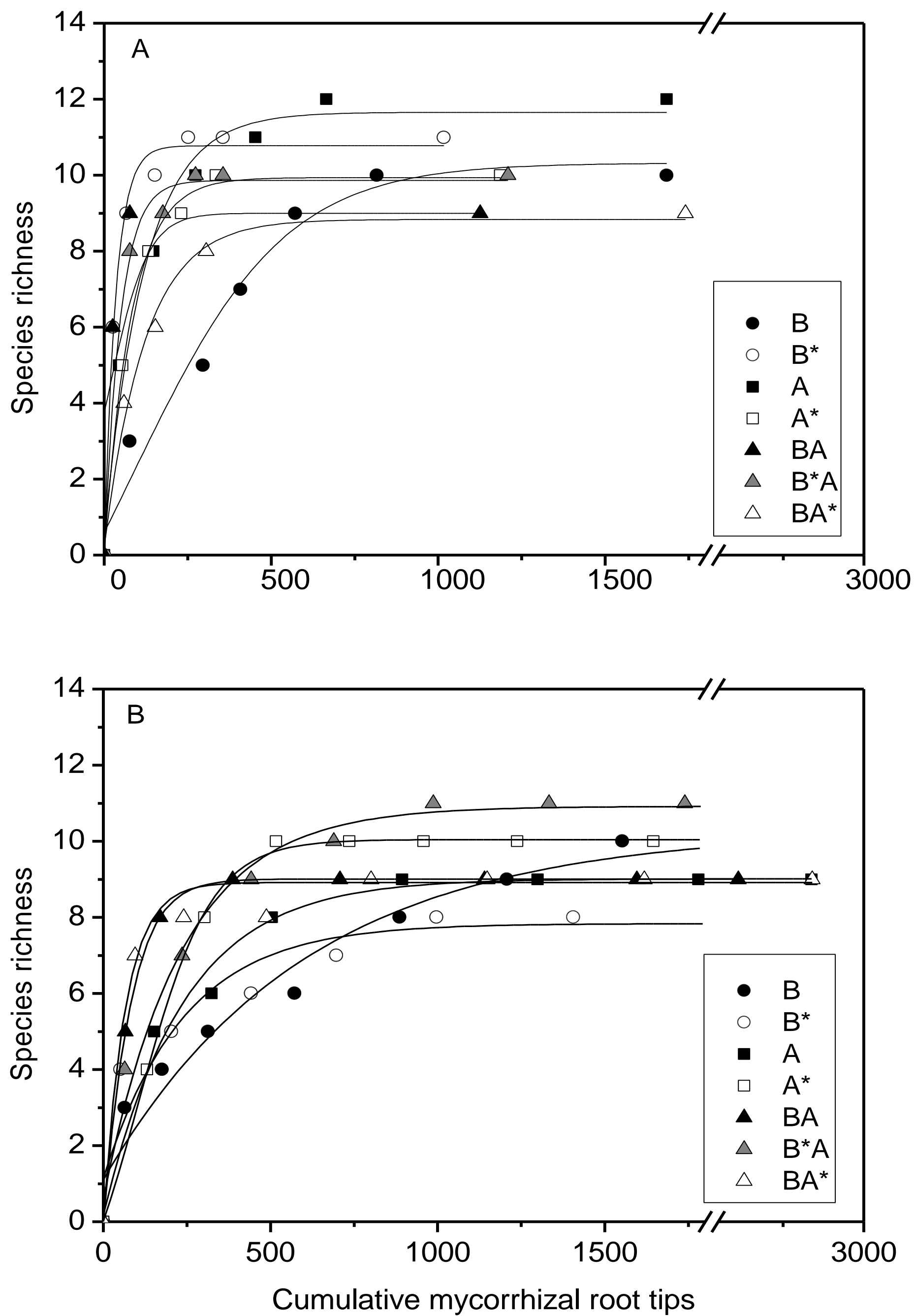


Figure 2: Cumulative ectomycorrhizal species richness in soil subjacent to different litter treatments in May (A) and in October (B), respectively. Species richness was determined five and eleven months after the following litter treatments: B = beech litter forest, B* = Beech litter greenhouse, A = Ash litter forest, A*= Ash litter greenhouse, BA= mixed litter beech + ash forest, B*A= mixed litter beech greenhouse + ash forest, BA* = mixed litter beech forest + ash greenhouse

2.4.2 Leaf litter affects mycorrhizal community structures

Seasonal and treatment-related changes in the EcM community structure were analyzed by Nonmetric Multidimensional Scaling (NMDS; Fig. 3). The first coordinate separated the EcM communities found in May and October (Fig. 3). In May no differences in the EcM community structures subjacent different leaf litter treatments were found. However, in October a clear separation (2nd coordinate) between EcM communities below mixed and mono-litter was observed. This division was apparently unrelated to the amounts of released nitrogen or the extent of leaf litter degradation because these environmental variables were aligned with the first and not with the 2nd coordinate (Fig. 3).

To investigate the changes in EcM community structure, we determined the difference in species abundance between October and May and calculated means across all treatments (Fig. 4). These data show that the abundances of *Clavulina* sp and *Tomentella castanea* were strongly decreased whereas those of *Russula ochroleuca, Xerocomus pruinatus* and a *Thelephora* sp (MT5) were strongly increased in October compared to May (Fig. 4).

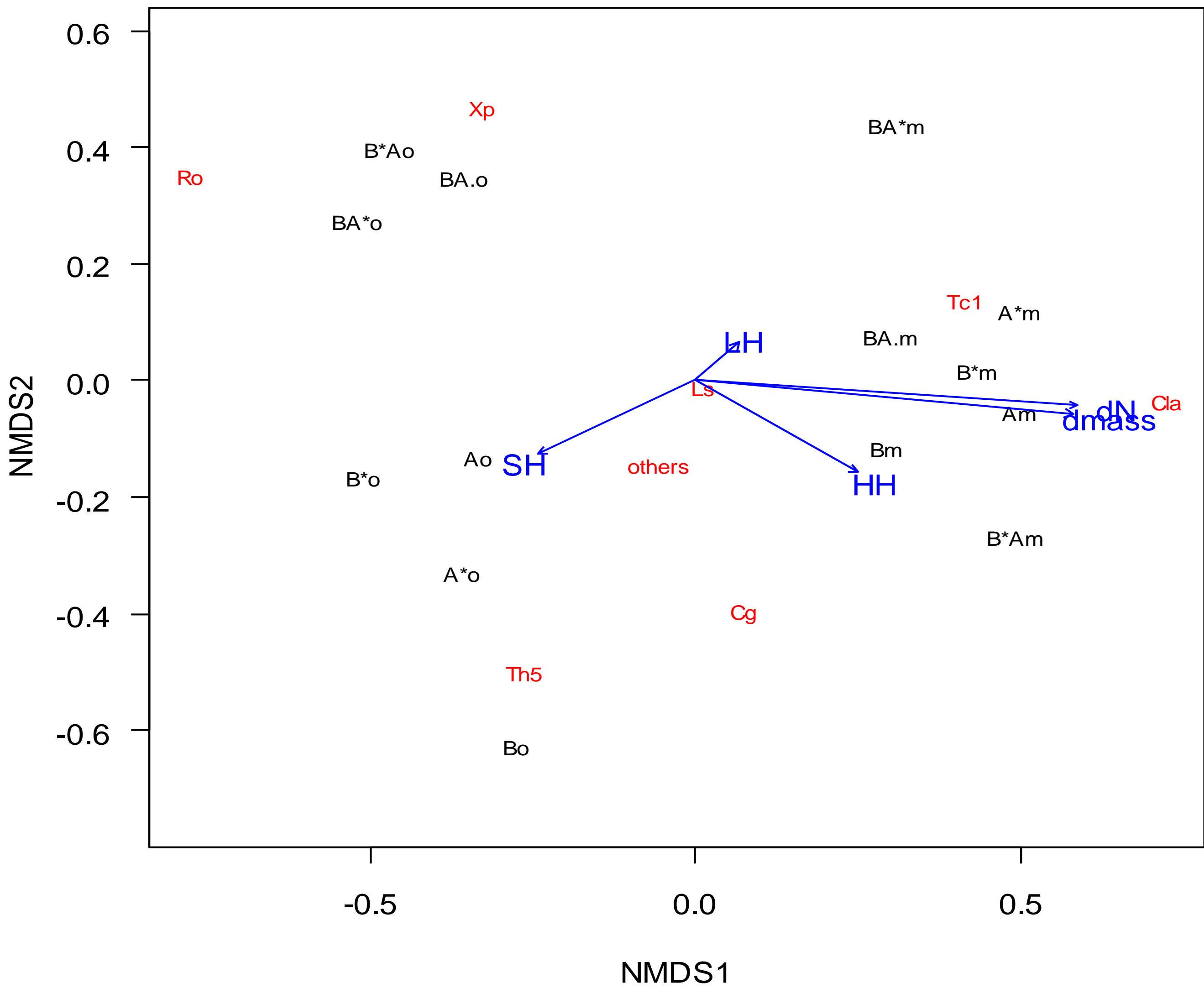


Figure 3: Nonmetric Multidimensional Scaling (NMDS) of EcM fungal community with season and litter treatment. The analysis was based on the relative abundance of the most abundant EcM species. Cla= ***Clavulina*** **sp., Tc1 =** ***Tomentella castanea*****, Cg=** ***Cenococcum geophilum*****, Ls =** ***Lactarius subdulcis*****, Th5 =** ***Thelephora*** **spec., Xp=** ***Xerocomus pruinatus*****, Ro=** ***Russula ochroleuca*****. B = beech litter forest, B* = Beech litter greenhouse, A = Ash litter forest, A*= Ash litter greenhouse, BA= mixed litter beech + ash forest, B*A= mixed litter beech greenhouse + ash forest, BA* = mixed litter beech forest + ash greenhouse. m = first harvest may, o= second harvest october**

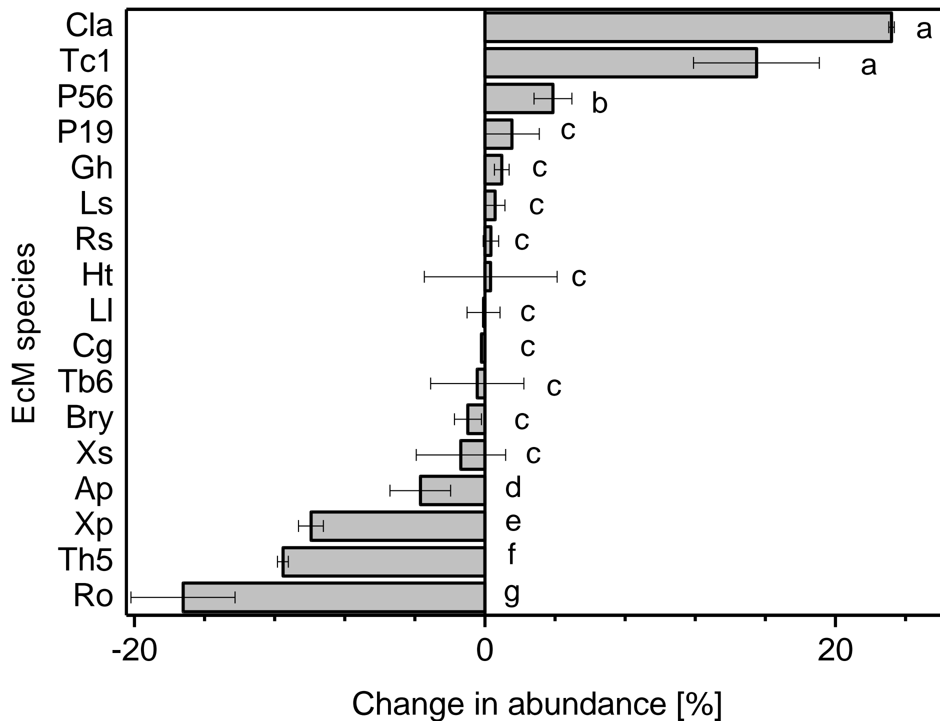


Figure 4: Changes in EcM fungal community structure (change in relative abundance [%] ± SE) between May and October, five and eleven months after start of the experiment. Cla = *Clavulina* sp., Tc1 = *Tomentella castanea*, P56= *Pezizales* 56, P19 = *Pezizales* 19, Gh= *Genea hipidula*, Ls = *Lactarius subdulcis*, Rs = *Russula* spec., Ht = *Hydnotrya tulasnei,* Ll = *Laccaria laccata*, Cg= *Cenococcum geophilum*, Tb6 = *Tomentella badia*, Bry = *Bryssocorticium atrovirens*, Xs = *Xerocomus* spec., Ap = *Amanita rubescens*, Xp= *Xerocomus pruinatus*, Th5 = *Thelephora* spec., Ro= *Russula ochroleuca*. Different letters indicate significant differences at p=0.05.

We classified the EcM fungal species according to their exploration types (see supplement Table S1) and analyzed the exploration type composition by Principle Component Analysis (PCA; Figure 5). The first component explained 48.1% of the variation and was determined by contact (+ direction) and medium distance (- negative direction) exploration types (Fig. 5). The EcM community in May was dominated by short distance exploration types. The second component explained 18.2% of the variation and was determined by short distance (+ direction) exploration types. In October, the EcM communities under mixed litter treatments were dominated by contact and by long distance exploration types, whereas the EcM communities under pure ash or beech litter were dominated by medium distance exploration types (greenhouse litter of pure ash and beech; A*o and B*o treatment) and short distance exploration types (forest leaf litter; Fig. 5).

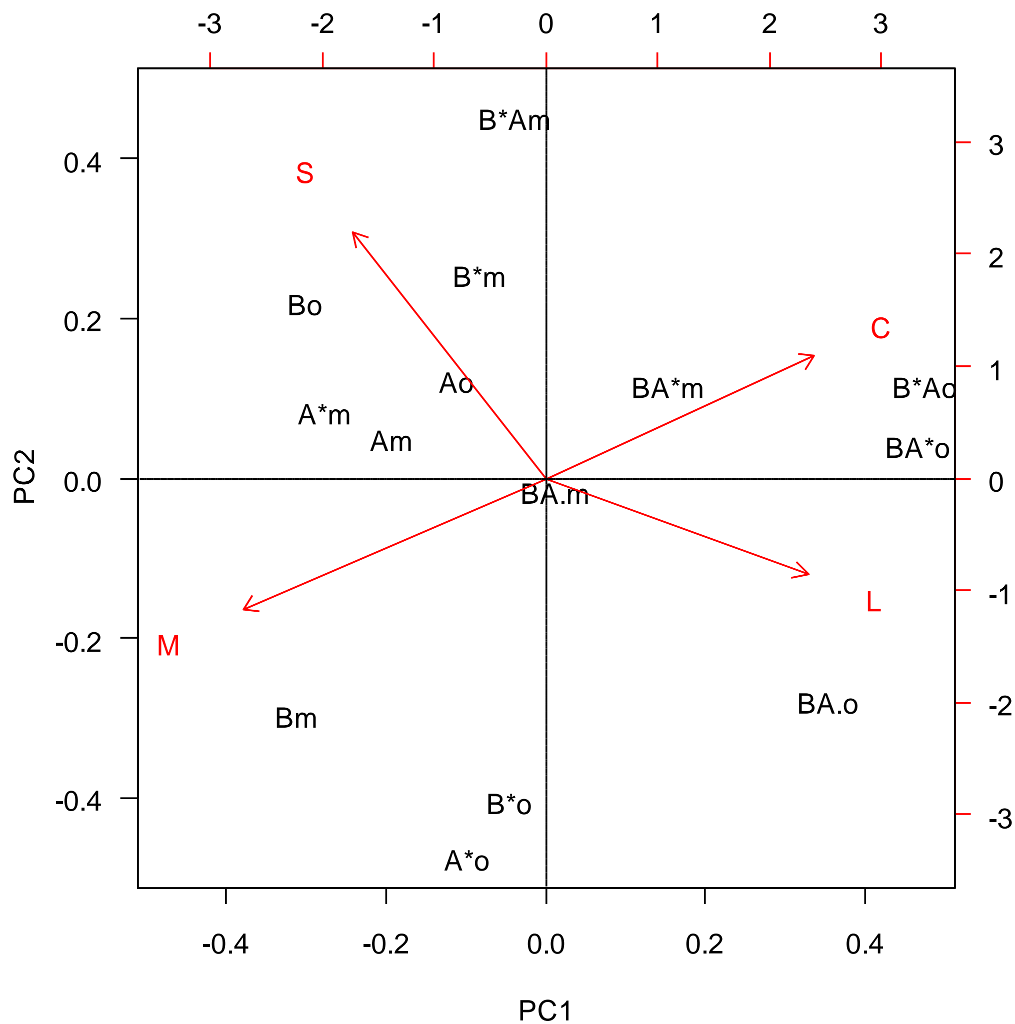


Figure 5: Principle Component Analysis (PCA) of composition of EcM exploration types. L = Long distance exploration type, M = medium distance exploration type, S = short distance exploration type, C = contact exploration type. B = beech litter forest, B* = Beech litter greenhouse, A = Ash litter forest, A*= Ash litter greenhouse, BA= mixed litter beech + ash forest, B*A= mixed litter beech greenhouse + ash forest, BA* = mixed litter beech forest + ash greenhouse. m = first harvest may, o= second harvest october

We furthermore determined hyphal lengths in the soil compartment underneath the leaf litter, which was not directly accessible to the roots because of the underlying mesh (mesh size 50 µm). Although the yield of extracted hyphae was surprisingly small (hyphal lengths were only a few millimeters in 5 g of soil), it allows cross-comparisons between the different treatments and seasons. The hyphae lengths were higher in October than in May (p=0.006, F=7.84; Fig. 6). In both seasons, the lengths of extracted hyphae did not differ significantly between leaf litter species (p= 0.154, F = 1.90; Fig. 6).

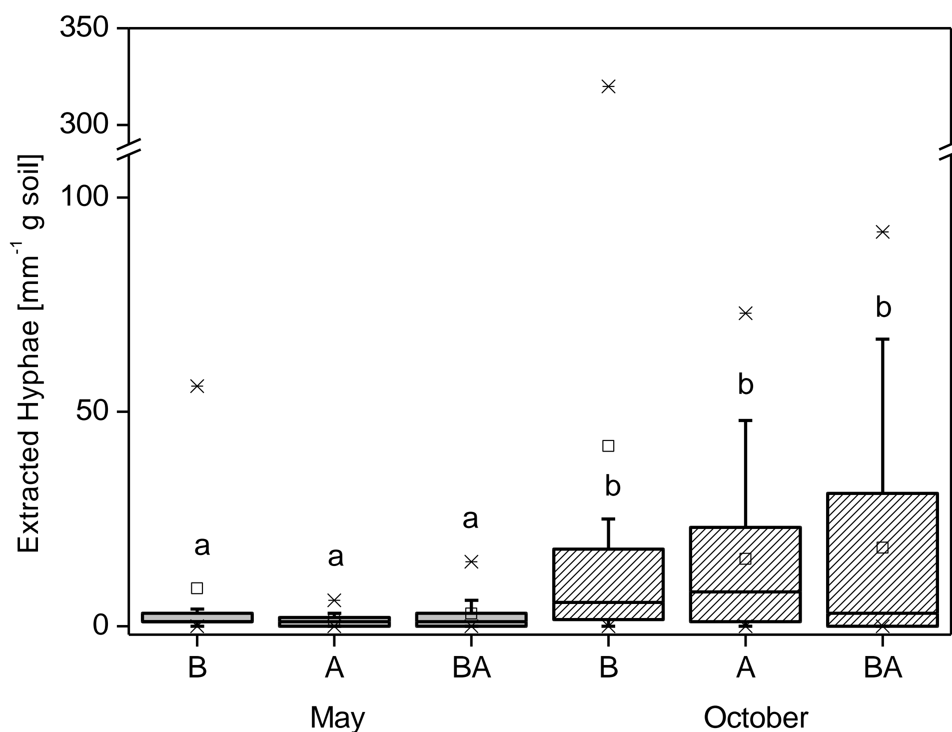


Figure 6: Hyphal length (mm / g soil) ± SE. The soil was collected in the soil compartment in the cylinder, which was not accessible by roots (n = 16 in ash and beech treatments, n= 24 in mixed treatments). Different letters indicate significant differences at p<=0.05. B = beech leaf litter, A = ash leaf litter, BA = mixed leaf litter (mixture of ash and beech leaf litter). Data are shown as box plots, with the box displaying the range of 25-75% of the data, the full square as the mean, the horizontal line as the median and the crosses as outliers.

2.5 Discussion

In our study, there was a shift in the EcM fungal community on beech trees after application of different leaf litter treatments containing ash leaf litter. Not only leaf litter species, but leaf litter parameter like N content are relevant factors. The ash litter grown in the greenhouse had a similar N content like beech litter, compared to the ash litter of the forest (Tab. 1). Decomposition rate of litter determines release rate of nutrients. There was a clear separation between the EcM communities of the first harvest in May and the second harvest in October, which was influenecd by nitrogen release and litter degradation rate. Different leaf litter treatments change litter quality, decomposition rate, and N availabilty and thus, are able to alter community composition of mycorrhizal fungi (e.g. Conn and Dighton 2000; Wallenda and Kottke 1998; Lilleskov et al. 2001, 2002; Peter et al. 2001). Removal of litter and humus can increase the number of EcM fungal species above- (fruitbodies) as well as belowground (mycelium; Smit et al. 2003). Fertilization with high inorganic N changes EcM fungal community composition (Kåren 1997; Wallenda and Kottke 1998; Peter et al. 2001; Lilleskov et al. 2001; 2002).

Other parameters like humidity are influenced by the overlying litter layer. In October, the EcM fungal communities differed between the pure leaf litter and the mixed leaf litter treatments and were obviously not related to the amounts of released nitrogen or the extent of litter degradation. Humidity of soil, humus and litter may be a relevant factor for this separation. Rainfall and soil moisture had a strong influence on community composition of EcM in a native Scots pine forest (Jarvis et al. 2013). Shi et al. (2002) showed that community composition as well as the abundance of different mycorrhizal types was changed after drought. Walker et al. (2005) reported a lower EcM fungal diversity at a dry forest site. However, other parameters like pH value or content of phenolic substances might also be related to this separation, but unfortunately these have not been examined in this study.

Community change of EcM fungal species in a pure beech forest as a result of different litter treatments was most pronounced in october, 10 months after start of the incubation. The individual EcM fungal species showed different reactions to applicated litter. To exclude seasonal effects, we refer to a long-term study by Lang et al. (2011) in the same area, where relative abundances of EcM fungal species over two years and seven sampling dates are reported.

Xerocomus has only rarely been detected on the first harvest (5 to 10 % relative abundance) which is in line with observations of Lang et al. (2011) reporting only relative abundance of *Xerocomus* of 1 to 2 % in the same forest area. In the mixed litter treatments in october, relative abundace of

Xerocomus species increased up to 30 %, probably because of changed nutrient availability. *Xerocomus badius* is an EcM fungus of high activity and element storage capacity (Kottke et al. 1998). In a study by Nygren et al. (2008), *Xerocomus communis* showed a high rate of mycelial growth on nitrate compared to over 100 other EcM fungal species. By contrast, *Cenococcum geophilum* seems to be preferentially associated with beech litter of the Hainich forest (relative abundance of 20 %), but was nearly not present in all other litter treatments. This species belongs to the common EcM fungal species in the Hainich forest and showed a long term average abundance on beech roots of 9.58 (±1.7) % (Lang et al. 2011). Since ash leaf litter changes soil parameters compared to pure beech leaf litter, this seems to alter conditions for growth of other EcM fungal species. Brearley et al. (2003) reported a lower percentage colonization by C*enococcum geophilum* after litter addition and suggested three possible reasons (1) *C. geophilum* is able to use organic nitrogen, (2) phenolics and volatiles affect the growth of *C. geophilum*, (3) *C. geophilum* is an EcM fungal species which is able to withstand desiccation and therefore has a competetive advantage compared to other EcM fungi.

Surprisingly, parameters like root biomass, mycorrhizal species richness, mycorrhizal infection rate or the proportion of dry root tips were not affected by leaf litter treatment in this study. Leaf litter addition led to a reduction in EcM fungal formation on Douglas fir (*Pseudotsuga menziesii*) (Rose et al. 1983) and red pine (*Pinus resinosa*) seedlings (Koide et al. 1998). Brearley et al. (2003) also reported lower EcM fungal diversity and Eveness when leaf litter was added.

We observed differences in occurance of exploration types between litter treatments. The upper soil horizon (0-5 cm) is usually dominated by contact exploration types (Cullings and Courty 2009 and reference therin). The increase of *Xerocomus* in the mixed leaf litter treatments in October also lead to a dominance of the long distance exploration type in these treatments. There is a hint that ability of EcM fungal species to exploit nutrient sources might be correlated with development of hyphae and rhizomorphs (exploration types). Hobbie and Agerer (2010) reported correlation of exploration types with nitrogen isotope natural abundance of EcM species.

In comparison to sandy soils, the method of hyphal extraction may be more difficult because of a high clay content of our soil (15 %, Guckland et al. 2009). Extraction of hyphae might be more difficult when hyphae are agglomerated with clay particles.

There was a clear effect showing that hyphal length was higher in October compared to May, but there was no significant effect between leaf litter species. However, length of hyphae appeared

longer in mixed leaf litter types of October, which is in line with the observation that this litter type is dominated by long distance exploration types, i.e. *Xerocomus* species (Fig. 3 and 5).

Production of EcM fungal mycelium (Söderström and Read 1987) and fruit bodies (Lamhamedi, Godbout and Fortin 1994) have been shown to be dependent on the current photoassimilates provided by the autotrophic plant. The mesh and the column itself form a barrier for mycorrhizal hyphae. If production of hyphae is not necessary because nutrients are easily accessable, formation of EcM mycelium might be suppressed. While Conn and Dighton (2000) found no significant differences regarding hyphal colonization between litter types (pine and oak), there are others studies where biomass of mycelium in soil is negatively influenced by soil nutrients (Wallander and Nylund 1992; Arnebrant 1994; Nilsson 2004, Nilsson et al. 2005, Hendricks et al. 2006). In a study of Nilsson and Wallander (2003), growth of EcM fungal hyphae was reduced to 50 % compared to an non-fertilized plot.

In this study, hyphae were much longer on second harvest date in october compared to first harvest in may. This could possibly be related to disturbance after setup of the experiment, where hyphae need some time to grow through the newly installed mesh barrier. Probably this is also related to a seasonal effect, because hyphae and fungi are most active in autum (Wallander et al. 2001; Hagerberg and Wallander 2002).

In conclusion, communities of EcM fungal species change due to an applicated leaf litter substrate. These changes seem to be related to changes in fungal hyphal length and exploration types. The reasons for this change are multiple and can not only be related to decomposition rate and nitrogen loss in the different leaf litter treatments, also humidity of soil, litter and humus is a relevant factor. There are general interspecific differences in the life strategy of EcM fungal species and in their ability to grow rapidly when conditions change and high amounts of nutrients are available. This has an impact on EcM fungal species dynamics.

2.6 Acknowledgements

We are grateful to the Deutsche Forschungsgemeinschaft (DFG) for financial support for the graduate school GRK 1086 “Die Bedeutung der Biodiversität für Stoffkreisläufe und biotische Interaktionen in temperaten Laubwäldern” (project A1). We thank the National Park administration for the permission to conduct our experiment in the area of the Hainich National Park. We thank Christina Langenbruch for providing leaf litter data, our colleagues of the graduate school and of the Department of Forest Botany for help with field sample collection and technical support and Thomas Klein for help with molecular work.

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Supplement: Table S1: Mycorrhizal species found in the study. Abundances of EcM species under different leaf litter treatments. Nitrogen content and litter degradation of different leaf litter treatments. C = Contact exploration type, L = Long distance exploration type, M = medium distance exploration type, S = short distance exploration type, n/a = not applicable. B = beech litter forest, B* = Beech litter greenhouse, A = Ash litter forest, A*= Ash litter greenhouse, BA= mixed litter beech + ash forest, B*A= mixed litter beech greenhouse + ash forest, BA* = mixed litter beech forest + ash greenhouse.

Supplementary table S1: Mycorrhizal species found in the study. Abundance of EcM species under different leaf litter treatments. Nitrogen content and litter degradation of different leaf litter treatments.

Full name	Accession number Genbank	Best BLAST hit (%) und accession number of hit	nucleotide fragment (R/L)	**Abbreviation**	Abundances of EcM species under different leaf litter treatments														**Exploration type**	**Reference for expl-type and N and mass**
					B	**B***	**A**	**A***	**BA**	**B*A**	**BA***	**B**	**B***	**A**	**A***	**BA**	**B*A**	**BA***		
Russula ochroleuca	KC952686	UDB000772 Russula ochroleuca (99 %)	679	Ro	0,0	0,0	0,2	0,0	0,0	6,9	0,0	9,8	13,6	15,5	6,9	21,4	30,0	30,5	C	Courty et al., 2008
Russula spec.	KC952679	UDB000901 Russula nigricans (97%)	691	Rs	0,0	0,0	0,0	0,0	4,0	0,0	0,2	0,0	0,0	0,0	0,0	0,0	1,8	0,0	C	Courty et al., 2008
Tomentella botryoides	KC952675	UDB000255 Tomentella botryoides (100 %)	583	Tb6	0,8	2,8	7,2	2,5	2,1	0,8	2,2	2,4	1,4	1,8	2,5	2,6	0,6	10,2	C	Agerer, 2001
Lactarius subdulcis	KC952680	UDB000048 Lactarius subdulcis (99%)	844	Ls	11,5	16,1	23,2	11,1	19,1	33,2	14,3	7,3	21,3	18,5	19,1	16,0	17,3	24,9	C	Agerer, 2001
Tomentella castanea	KC952674	UDB000120 Tomentella castanea (99%)	668	Tc1	19,7	31,9	13,9	21,3	23,9	15,9	30,9	7,8	7,3	10,5	3,7	9,2	8,9	1,6	C	Agerer, 2001
Xerocomus spec.	n/a	n/a	n/a	Xs	1,2	0,0	1,7	1,2	0,0	0,0	0,0	0,0	0,0	0,0	11,0	0,0	2,0	0,7	L	Agerer, 2001
Xerocomus pruinatus	KC952677	UDB000049 Xerocomus pruinatus (99%)	872	Xp	3,4	2,6	2,0	5,1	10,0	0,6	21,1	13,4	8,7	5,4	7,3	30,8	28,5	20,1	L	Agerer, 2001
Laccaria spec.	KC952691	UDB001495 Laccaria maritima (98%)	655	Ll	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,5	0,0	0,0	0,0	0,0	0,0	0,0	M	Courty et al., 2008
Amanita rubescens	n/a	n/a	n/a	Ap	0,0	1,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	2,2	11,0	7,8	0,7	5,5	M	Courty et al., 2008
Thelephora spec.	KC952710	FM995568.1 Uncultured Thelephora (99%)	857	Th5	29,6	7,6	7,8	7,3	12,2	2,8	8,4	33,6	44,0	33,0	30,5	8,4	2,4	4,5	M	Courty et al., 2008
uncultured basidiomycete (Bryssocorticium)	KC952692	UDB016321 Byssocorticium caeruleum (94 %)	950	Bry	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,6	0,0	0,0	0,0	0,0	6,2	0,0	S	Courty et al., 2008
uncultured Pezizales	KC952678	FN393151.1 uncultured Pezizaeae (99%)	690	P19	0,0	0,0	7,1	0,0	0,0	3,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	S	Courty et al., 2008
Genea hispidula	KC952681	UDB001408 Genea hispidula (99%)	1297	Gh	0,8	5,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	S	Agerer, 2001
Cenococcum geophilum	n/a	n/a	n/a	Cg	0,9	1,9	1,4	7,9	2,0	2,6	10,4	21,5	0,4	1,4	0,0	2,0	1,6	1,6	S	Courty et al., 2008
uncultured Pezizaeae	KC952688	FJ013079.1 uncultured ectomycorrhiza (Peziza) clone (100)	1284	P56	6,8	6,4	4,4	9,2	6,4	10,4	2,1	2,0	0,0	11,7	4,4	0,0	0,0	0,4	S	Courty et al., 2008
uncultured ECM (Clavulina)	KC952704	EU862208.1 Clavulina cf. Amethystina (99%)	1123	Cla	23,7	22,3	35,4	34,4	20,3	23,1	10,3	1,6	0,0	0,0	3,6	1,8	0,0	0,0	M	Courty et al., 2008
uncultured basidiomycete (	KC952707	GU327421.1 Uncultured Hydnotrya clone (95%)	1127	Ht	0,0	0,0	2,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	U	
Nitrogen content (mg per cylinder)				D N	-42	63	36	-11	-45	-22	-11	47	215	157	270	108	184	181		Langenbruch 2012
Litter degradation (% loss of litter mass)				D Mass	0,0	49,0	9,0	27,0	1,0	10,0	15,0	63,0	97,0	73,0	95,0	81,0	82,0	82,0		Langenbruch 2012

*Exploration type C = contact, M = medium rage, s = short distance, L = Long distance, u = unknown

CHAPTER

3

Ectomycorrhizal fungal species on root tips differ in nitrogen concentrations and ^{15}N accumulation from $^{15}N/^{13}C$ labelled tree leaf litter

3.1 Abstract

Ectomycorhizal (EcM) fungi are functional important components of forest ecosystems. Several EcM fungal species differ in their importance for nitrogen nutrition of trees. We conducted a field leaf litter decomposition experiment where ^{15}N/^{13}C labelled leaf litter mixtures of beech (*Fagus sylvatica* L.) and ash (*Fraxinus excelsior* L.) were applied in an old growth deciduous beech forest in the Hainich National Park, Thuringia, Germany. ^{15}N assimilation and N concentration of roots and EcM fungal species on root tips in the mineral soil horizon (5-10 cm soil depth) were determined. Fungi and roots assimilated ^{15}N over a minimum distance of 5 cm from the labelled leaf litter; the assimilation rate increased between the two harvest dates, five and eleven months after the start of the decomposition experiment. This study revealed significant differences in N concentration as well as ^{15}N assimilation rate between roots and several EcM fungal species on root tips in the mineral horizon of a beech forest stand. Whether high ^{15}N accumulation rate is correlated with N concentration of the EcM fungal species and whether the observed differences between EcM fungal species are related to functional attributes (exploration types) is discussed. Roots accumulated ^{15}N from labelled leaf litter, following a sigmoidal curve, with saturation on second harvest in October. There was a slight, but not significant relationship between N release from leaf litter and root mass production after eleven months. Stable isotope measurement of EcM fungal species colonizing root tips is possible and provides an important additional view on nutritional aspects of EcM fungi, which were fruitbody-focused for a long time.

3.2 Introduction

One of the main nitrogen sources in forests is decaying leaf litter (Bending and Read 1995; Perez-Moreno and Read 2000). Early studies of Bending and Read (1995) showed mobilization of N from patches of organic material by the ectomycorrhizal (EcM) fungi *Suillus bovinus* and *Thelephora terrestris.* Now it is well accepted that mycorrhizal fungi play an important role in leaf litter decomposition and mobilization of nitrogen (N) from complex organic sources which are otherwise unavailable to host roots (Talbot et al. 2008, 2013; Cullings and Courty 2009; Pena et al. 2013).

The functional roles of EcM fungal species for acquisition of litter-derived N are not well understood. There were attemps to classify EcM fungal species according to functional attributes, for example protein vs. non-protein fungi (Abuzinadah and Read 1986), organic N use (Gebauer and Taylor 1999; Lilleskov et al. 2002; 2011), late- vs. early-stage fungi (Newton 1992), or according to exploration types, mycelial distribution, development of rhizomorphs and hydrophobic vs. hydrophilic properties (Agerer 2001). It is widely acknowledged that some, but not all EcM fungal species exhibit some abilities to sequester nutrients from natural substrates and are able to mobilize N directly from leaf litter and soil organic matter (Perez-Moreno and Read 2000) since EcM fungi are known to produce the necessary enzymes and show different enyzme activity profiles (Cullings and Courty 2009; Pritsch and Garbaye 2011; Tedersoo et al. 2012). EcM fungal species differ in functional traits like nutrient uptake and transfer capacities (Burgess et al. 1993), because of root colonization extent and length of hyphae in soil. In the concept of exploration types (Agerer 2001) for example long distance exploration types (LD) are able to transport nutrients over larger distances in the soil than contact or short distance exploration types. This assumption was supported by ^{15}N stable isotope analysis, where sporocarps of different exploration types differed in ^{15}N stable isotope signature, with the hydrophobic LD exploration type showing high ^{15}N values (Hobbie and Agerer 2010). Functional differences between EcM fungal species might be reflected by differences in N concentration in the mycorrhizal tissues. Hobbie et al. (2008). found that ^{15}N and N % in EcM root tips weres highly correlated in an ammonium treatment, but not in nitrate treatment.

It is known that EcM species, even strains, differ in their natural δ^{15}N natural abundance with inter- and intraspecific variation in ^{15}N signatures (Taylor and Fransson 2006; Trudell et al. 2004); this may reflect N transport and transformation of N-compounds (fractionation) as well as the N source (mineral or organic substrates). Most ^{15}N studies about mycorrhiza used sporocarps (Hobbie and Högberg 2012), only few studies investigated N concentrations and N assimilation rates of EcM fungi on root tips under natural conditions in a forest ecosystem (Högberg et al. 1996; Zeller et al.

2000; 2007; Haberer et al. 2007; Tedersoo et al. 2012; Pena et al. 2013; Albarracin et al. 2013). Jones et al. (2009) injected in a greenhouse experiment ^{15}N labelled nitrate, ammonium or aspartate around seedlings and found differences in ^{15}N accumulation between seedlings colonized by EcM species *Wilcoxina* sp. and *Cenococcum* sp. Only few studies if this pattern can be extended to the forest and if EcM species on root tips differ in N assimilation rates from natural substrates like leaf litter (Tedersoo et al. 2012, Pena et al. 2013).

We investigated EcM fungal species on root tips in a leaf litter incubation experiment in a deciduous old-growth forest in Thuringia, Germany. The aim of this study was to trace the uptake of ^{15}N into EcM root tips and roots from different mixtures of leaf litter (beech [*Fagus sylvatica* L.] vs. ash [*Fraxinus excelsior* L.] tree leaf litter) in a pure beech stand by different ectomycorrhizal (EcM) fungi within their natural communities. We wanted to find out whether N concentrations and ^{15}N uptake patterns of the EcM fungal species were affected by different leaf litter treatments of beech and ash leaf mixtures.

We hypothesized that EcM fungal species in a beech forest are functional diverse and therefore

- differ in N concentrations, which is a species-specific trait and therefore not influenced by application of different leaf litter treatments.
- show interspecific differences for ^{15}N acquisition from different leaf litter substrates. ^{15}N accumulation is related to fungal exploration types.
- ^{15}N from labelled leaf litter is transferred by EcM fungal species to roots, which leads to increasing ^{15}N accumulation in fine roots. Different N release from leaf litter treatments influences total root biomass.

3.3 Materials and methods

3.3.1 Labelled leaf litter

Labelled leaf litter was produced in a greenhouse for one vegetation period (09.04.2008 to 14.10.2008) using one-year old beech (*Fagus sylvatica* L.) and ash (*Fraxinus excelsior* L.) plants. A Hoagland–based nutrient solution containing ^{15}N was used for irrigation (0.6 mM $CaCl_2*2H_2O$, 0.04 mM $MgSO_4*7H_2O$, 0.01 mM $FeCl_3*6H_2O$, 0.4 mM KH_2PO_4, 1.8 µM $MnSO_4*H_2O$, 0.064 µM $CuCl_2*2H_2O$, 0.15 µM $ZnCl_2$, 0.1 µM MoO_3, 0.01 mM H_3BO_3, 2 mM $^{15}NH_4{}^{15}NO_3$ [Euriso–top, Saint–Aubin, Essonne, France]). The soil moisture was kept at 30% during the growing season. $^{13}CO_2$ was applied to the greenhouse. The CO_2 concentration of the air was 1018 ppm ± 340. Temperature was kept at 22.8°C ±2.8 and humidy at 71.8 % ±13. The plants were grown with supplementary light under long-day conditions (16 h light, 8 h dark) achieving 85 ±30 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR) at plant height. At the end of the vegetation period, leaves were removed from the plants, air-dried and stored in paper bags at room temperature. Unlabelled leaf litter of ash and beech was collected in the Hainich forest in autumn 2008, air-dried and stored in paper bags at room temperature.

3.3.2 Leaf litter treatment

The leaf litter treatment was conducted in the Hainich forest in Thuringia, Germany which is unmanaged for at least 40 years. Annual precipitation is 670 mm and the annual mean temperature is 7.5 °C, respectively (Leuschner et al. 2009). In the experimental plot the soil is classified as luvisol; the geological substrate is limestone covered by loess and the pH (H_2O) of the soil was 4.2 - 4.4 (Guckland et al. 2009).

In a monospecific 100 to 200-year-old beech stand (51°05’28’’N, 10°31’24’’E) an experimental plot (50x50m) was enclosed by a fence. The leaf litter treatment was started in December 2008 by exposing soil filled plastic cylinders with a diameter of 24 cm and a height of 20 cm placed in the forest soil. All cylinders were inserted 5 cm into the soil, maintaining the different soil layers. Seedlings were removed, if present. To prevent root to grow inside the cylinder, a nylon mesh (mesh size 40 µm) was fixed under the bottom side of the plastic cylinders; therefore, only ingrowth of hyphae was possible. The original leaf litter inside the cylinders was removed and replaced by 14.38 g of either unlabelled or $^{13}C/^{15}N$-labelled leaf litter, resulting in seven leaf litter treatments (Table 1), which were repeated eight times each. This resulted in a total of 56 cylinders per harvest date (2 harvest dates). Within four subplots, the cylinders with the seven different litter treatments were randomly distributed.

Tab. 1: Seven litter treatments used in the litter decomposition experiment and release of ^{15}N ± SE from leaf litter. Asterisks (*) indicate labelled litter types

			Release from leaf litter [µg ^{15}N ± SE]			
			May		October	
B	beech	unlabelled	-0.16	±0.04	0.17	±0.02
B*	beech*	labelled	2.05	±0.31	3.70	±0.23
A	ash	unlabelled	0.14	±0.04	0.58	±0.01
A*	ash*	labelled	6.68	±3.79	25.93	±0.88
BA	beech+ash	unlabelled	-0.16	±0.05	0.40	±0.03
B*A	beech*+ash	beech labelled + ash unlabelled	0.27	±0.14	1.80	±0.13
BA*	beech+ash*	beech unlabelled + ash labelled	2.55	±1.55	9.53	±0.39

3.3.3 Harvest and sample collection

Harvests took place 5 months (May 5th 2009) and 11 months (October 20th 2009) after the start of the leaf litter treatment in December 2008. Six samples (5x5 cm, of a total soil volume of approximately 100 cm³) were taken under the cylinder in a soil depth of 5-10 cm (including fine roots and mycorrhizal root tips). Fine roots were analysed to determine the number of root tips and isotope composition of fine roots and mycorrhizal root tips were used for analysis of the EcM community and isotope composition of mycorrhizal species. All samples were stored at 4 °C until further analysis.

At harvest, the remaining leaf litter on the surface of the soil cores inside the cylinders was collected. Fresh and dry mass of the leaf litter were determined for each cylinder. Leaf litter was air-dried and was used to determine (^{15}N, ^{13}C), N and C content of the leaf litter (Langenbruch et al. 2013).

3.3.4 Analysis of roots and mycorrhizal root tips

Roots were carefully washed to remove soil particles, and were sorted into living non-mycorrhizal, mycorrhizal and dead root tips.

Mycorrhizal root tips were observed in petri dishes under a binocular (Stemi SV 11, Zeiss, Jena, Germany) and morphotypes of EcM fungi were determined after morphological and anatomical characteristics (for details, http://www.uni-goettingen.de/de/goe-fungi/92389.html). Number of root tips were counted.

Ectomycorrhizal root tips stored at -80 °C were ground in a mill (Type MM2, Retsch, Haan, Germany). For DNA extraction, the DNAeasy Mini Plant Kit (Quiagen, Hilden, Germany) was used. ITS sequencing using the fungal specific primer pair ITS1F and ITS4, cloning, Gene Bank

and UNITE comparisons were performed as previously described in Lang et al. (2011). The sequences have been deposited in NCBI GenBank with the GenBank accession numbers: KC952674-KC952675, KC952677-KC952681, KC952686, KC952688, KC9526 91-KC952692, KC952704, KC952707 and KC952710.

3.3.5 Isotopic and elemental analysis

Fine roots and mycorrhizal root tips of selected EcM fungal species present in sufficient amounts (0.7 to 1 mg of each fungal species or root material) were separated, collected in reaction tubes and freeze-dried for five days (vacuum; -60°C; P4K-S; Dieter Piatkowski, München, Germany). Subsamples of the material were weighed in preweighed tin capsules (5x9 mm, IVA Analysentechnik, Meerbusch, Germany) for total ^{14}N, ^{15}N, ^{12}C and ^{13}C isotopic analysis. Isotopic and elemental analysis was carried out using an elemental analyzer (Heraeus Elementar Vario EL, Hanau, Germany), coupled to an isotope ratio mass spectrometer (delta Plus, Finnigan MAT, Bremen, Germany). The N concentration in fine roots and mycorrhizas was expressed as the percentage of N per dry mass of the sample. The ^{15}N abundance is expressed in delta units, and denotes parts per thousand (‰) using the ratio ^{15}N:^{14}N in atmospheric N as the standard. Atmospheric delta ^{15}N is 0 ‰ by definition. The internal standard was acetanilide, calibrated against atmospheric N (N_2). Stable isotope abundances are reported as delta ^{15}N (‰) = 100* ($[R_{sample} – R_{standard}] / R_{standard}$).

^{15}N Atom Percent Excess (APE) was calculated as: ^{15}N Atom % (sample) – ^{15}N Atom % (control, natural abundance). ^{15}N in root mass [µg APE] was calculated as: (^{15}N [µg APE] / g root dry mass [DM]) * root dry mass [g] per cylinder. Relative contribution of the EcM fungal species i to ^{15}N accumulation in mycorrhiza was calculated for each leaf litter type and for each harvest date as : µg ^{15}N / g dry mass [DM] in EcM fungal species i * relative abundance of EcM fungal species i.

3.3.6 Statistical analysis

Eight cylinders per leaf litter treatment were harvested for mycorrhiza observation and analysis. Statistical analysis was conducted in R, version 2.10.0 (R Development Core Team, 2009; http://www.r-project.org/). Where appropriate, data are indicated as means ± SE. ANOVA followed by Tukey's HSD test was conducted to detect significant differences between leaf litter treatments and fungal species. Treatment effects were considered significant for p values ≤ 0.05.

Linear regression was done using Origin Pro 8.5G (Origin Lab Corp., Northhampton, USA) to examine the significance of delta ^{15}N values for EcM fungal species and roots in response to ^{15}N

release in leaf litter treatments. Fitted datasets of linear regressions of EcM species were compared using Origin Pro 8.5G.

3.4 Results

3.4.1 EcM fungal species on root tips differ in N concentration, which is not influenced by leaf litter treatments

Nitrogen concentration [% N] was measured in Ecm fungal species and in fine roots. N concentrations in EcM fungal species and roots were lower in October (2.31 % in EcM fungal species and 1.54 % in roots) than in May (2.52 % in EcM fungal species and 1.76 % in roots, respectively; $p=0.02$). There were significant differences in N concentrations between EcM fungal species and roots ($p<0.001$; Fig. 1); however no influence of the leaf litter treatment ($p= 0.14$). While *Lactarius* (Ls) and *Xerocomus* (Xs) showed higher N concentrations compared to other EcM fungal species on root tips, *Amanita* (Ap) or *Thelephora* (Th5) displayed rather lower N concentrations compared to the other EcM species (Fig.1). Roots exhibited lower N concentrations compared to EcM fungal root tips, except for *Thelephora* (Th5).

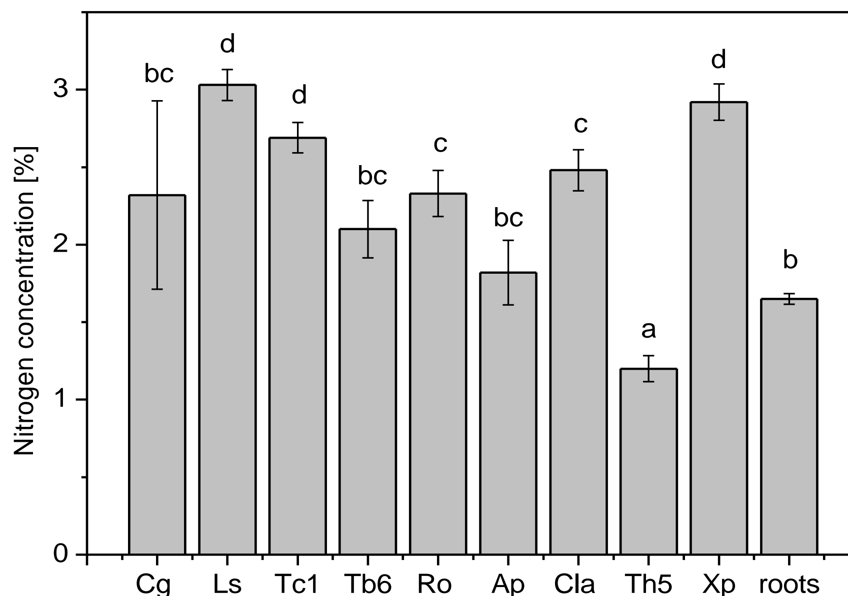


Fig. 1 Nitrogen concentration [%] in EcM fungal species and roots. Cg=*Cenococcum geophilum*, Ls=*Lactarius subdulcis*, Tc1=*Tomentella castanea*, Tb6=*Tomentella badius*, Ro=*Russula ochroleuca*, Ap= *Amanita rubescens*, Cla=*Clavulina* spec., Th5=*Thelephora* spec., Xp=*Xerocomus pruinatus*, roots= fine roots. Different letters indicate significant differences at p<0.05.

3.4.2 Differences in 15N content and litter degradation are reflected by 15N accumulation in roots and EcM fungal root tips

The four labelled leaf litter treatments differed in the amount of released ^{15}N (Table 1); this pattern was reflected in ^{15}N accumulation in EcM fungal species. EcM fungi and roots received ^{15}N labelled compounds from differently labelled leaf litter over a minimum distance of 5 cm. ^{15}N in EcM fungal tissues on root tips and in roots increased between the two harvests in May and October (Fig.2). Correlation between ^{15}N released from the labelled leaf litter and the ^{15}N accumulated in roots EcM fungal species showed interesting patterns. ^{15}N accumulation was higher on the second harvest in October compared to the first harvest in May (Fig. 2). EcM fungal species showed a positive linear relationship between ^{15}N released from the labelled leaf litter and the ^{15}N accumulated in the EcM fungal tissues (Fig. 2; Table 2). Roots showed markedly lower ^{15}N accumulation compared to ^{15}N accumulation in EcM fungal species (Fig. 2).

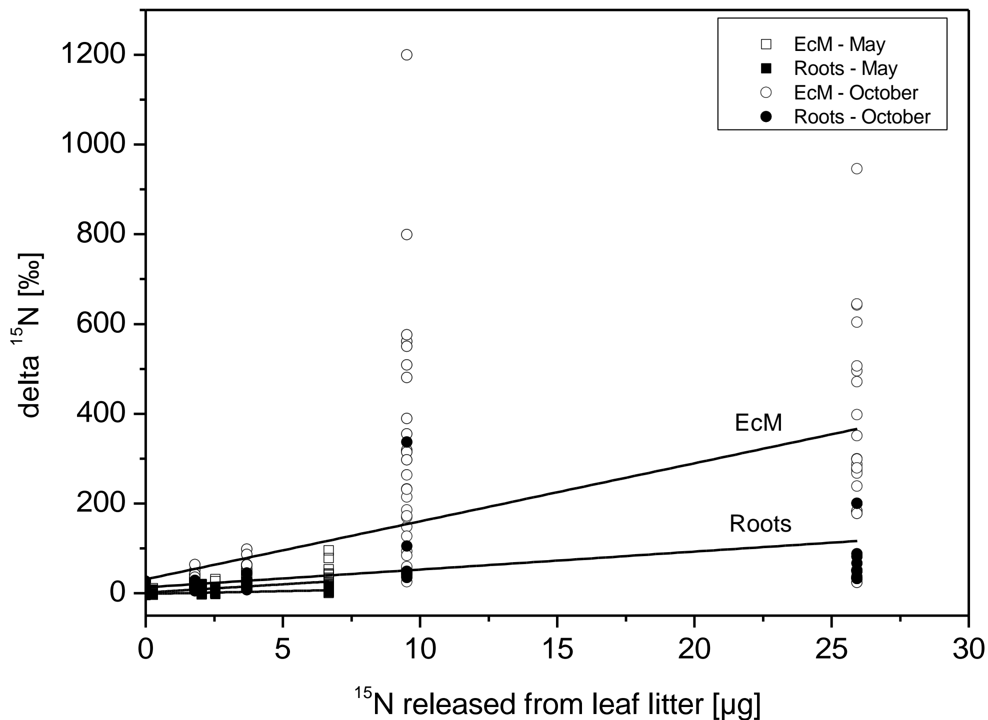


Fig. 2: Linear regression of δ ^{15}N [‰] in roots and in EcM fungal species on root tips (EcM) vs. ^{15}N released from leaf litter [µg] in May and October. Regression coefficients and significance levels are shown in Table 2.

3.4.3 Interspecific differences in 15N enrichment of EcM fungal species

EcM fungal species differed in ^{15}N accumulation from the labelled leaf litter (Fig. 3; Table 2). *Lactarius subdulcis* (Ls) accumulated high amounts of ^{15}N in lower labelled litter treatments in both harvest dates (Fig. 3 A, C) and *Russula ochroleuca* (Ro), *Clavulina* spec. (Cla) and *Thelephora* spec. (Th5) accumulated much ^{15}N in lower labelled litter treatments in October (Fig. 3 C,D). In May, Th5 (slope 8.06) showed the highest accumulation, followed by Tc1 (4.01) ≈ Cla (3.97) > Roots (1.25) ≈ Ls (0.91). In October, Xp (slope 37.15) displayed the highest accumulation of ^{15}N, followed by Ro (21.75) > Ls (15.70) ≈ Ap (14.96) > Th5 (8.98) > Tc1 (4.29) > Roots (3.99). Remarkably, Th5 and Tc1 showed similar ^{15}N accumulation on both harvests. Comparison between linear regression lines of EcM species revealed significant differences between roots and all measured EcM fungal species, except for Tc1 in October (p=0.97; Table 3). In May, regression lines between Th5 and Ls, Tc1 and Cla differed significantly (Table 3), and as well as Cla and Ls. In October, regression line between Tc1 and Ro, Cla and Xp differed significantly, as well as between Tc1 and Ls (Table 3).

We wanted to find out whether differences for ^{15}N enrichment are related to the N concentration of the EcM fungi and we correlated the slope of regression line with the N concentration in EcM species (Fig.4). We found no general pattern that species with high N concentration also accumulate high amounts of ^{15}N from leaf litter (Fig. 4). While Ls and Xp showed higher N concentrations compared to other EcM fungal species on root tips (Fig.1), which is in line with high ^{15}N accumulation (Fig 3), other species like Ap or Th5 displayed rather low N concentrations (Fig.1). Th5 showed the lowest N% of all EcM species but high ^{15}N accumulation. Roots displayed low N concentration compared to EcM (Fig. 1) and low ^{15}N accumulation (Fig.2).

To investigate the relative contribution of different EcM species to N uptake, their specific ^{15}N enrichment was weighed by their relative abundance. ^{15}N assimilation [µg/g] were combined with the relative abundance of each species per leaf litter treatment and harvest (Fig.5). *Lactarius* (Ls) contributed comparably stable to ^{15}N uptake in all leaf litter treatments and harvest dates, except a low contribution in the A* treatment in May (2.54 % relative contribution vs. 11.1 % relative abundance; Supplementary Table S1 and S3). *Xerocomus* showed a relatively high contribution to ^{15}N uptake in October (32.24 %; Table S3), as well as *Russula* (Ro; 22.99 %). *Clavulina* (Cla; 26.40 %) contributed much to ^{15}N uptake in May (Table S3).

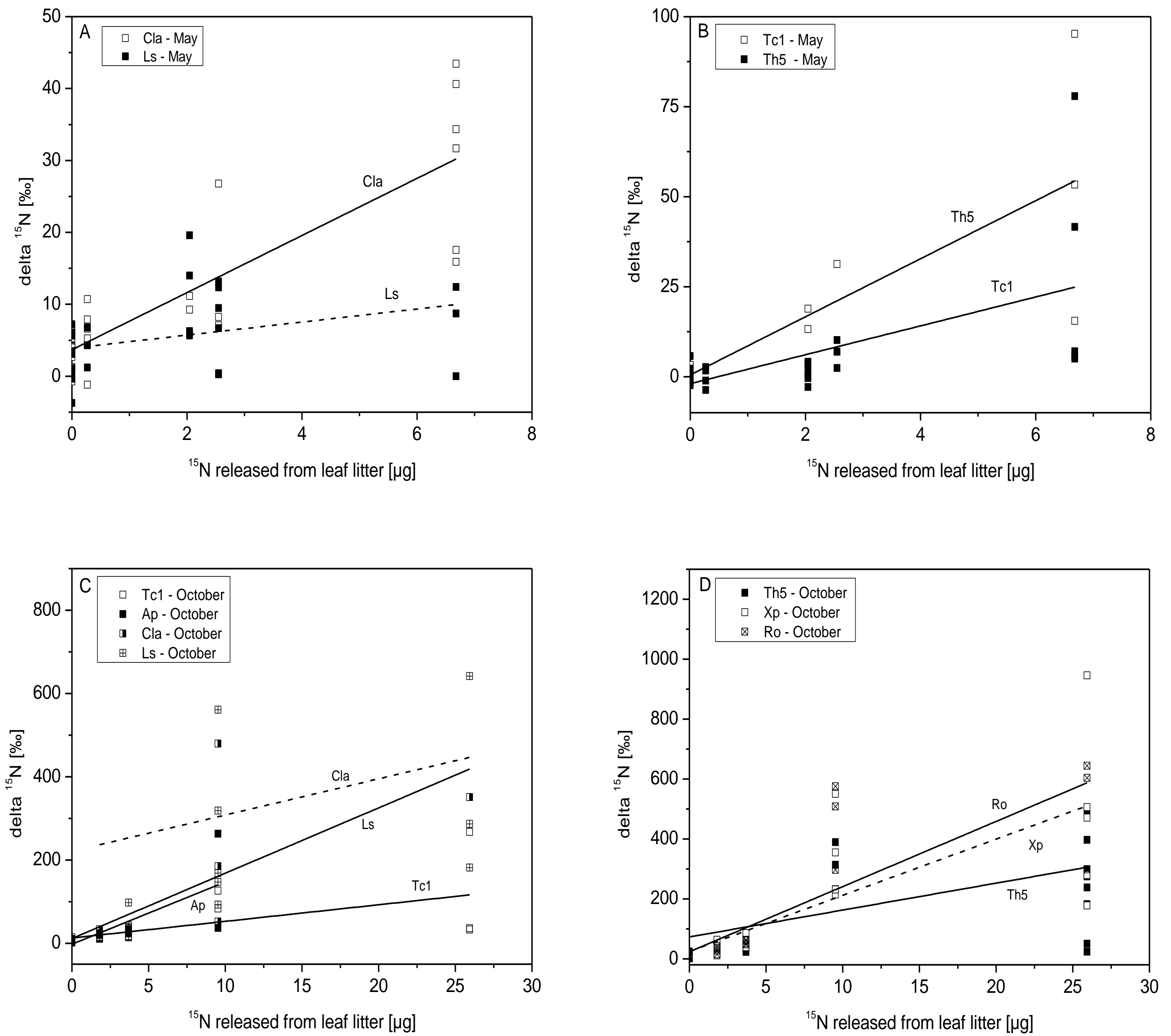


Fig. 3 A-D: Linear regression of δ ^{15}N [‰] in roots and in different EcM fungal species on root tips vs. ^{15}N released from leaf litter [µg]. A,B = May data C,D = October data. Ap= ***Amanita rubescens*****, Ls=*****Lactarius subdulcis,*** **Tc1=*****Tomentella castanea*****, Ro=*****Russula ochroleuca*****, Cla=*****Clavulina*** **spec., Th5=*****Thelephora*** **spec., Xp=*****Xerocomus pruinatus*****, Roots=fine roots without mycorrhiza. Regression coefficients and significance levels are shown in Table 2.**

Table 2: Parameters of regression lines (Fig. 2 and 3) of δ ^{15}N [‰] in roots and in EcM fungal species on root tips (EcM) vs. ^{15}N released from leaf litter [µg] in May and October. Significant differences at p<0.05 are marked in bold letters. EcM= all EcM fungal species summarized, Roots=roots without mycorrhiza. Cg=*Cenococcum geophilum*, Ls=*Lactarius subdulcis*, Tc1=*Tomentella castanea*, Ro=*Russula ochroleuca*, Ap= *Amanita rubescens*, Cla=*Clavulina* spec., Th5=*Thelephora* spec., Xp=*Xerocomus pruinatus.* n=number of samples.

Species	Harvest	Intercept	Slope	p	F	R²	n
EcM	May	1.46	3.68	**1.01 E-12**	64.96	0.37	112
EcM	October	30.97	12.93	**4.44 E-10**	82.55	0.34	157
Roots	May	-1.67	1.25	**4.02 E-6**	27.53	0.37	47
Roots	October	12.88	3.99	**1.35 E-5**	23.62	0.32	49
Cla	May	3.68	3.97	**4.22 E-10**	84.66	0.74	31
Cla	October	221.19	8.70	0.65	0.24	0.18	6
Ls	May	3.90	0.91	0.07	3.53	0.09	27
Ls	October	11.44	15.70	**1.41 E-6**	37.17	0.56	30
Th5	May	0.51	8.06	**3.19 E-4**	23.49	0.62	15
Th5	October	72.88	8.98	**0.03**	4.94	0.12	29
Ro	October	23.47	21.75	**3.33 E-7**	53.55	0.70	23
Tc1	May	-1.92	4.01	**8.71 E-4**	14.14	0.33	28
Tc1	October	12.65	4.29	**0.002**	12.70	0.37	21
Ap	October	-2.13	14.96	**0.03**	7.10	0.43	9
Xp	October	24.15	37.15	**3.23 E-6**	37.15	0.60	25

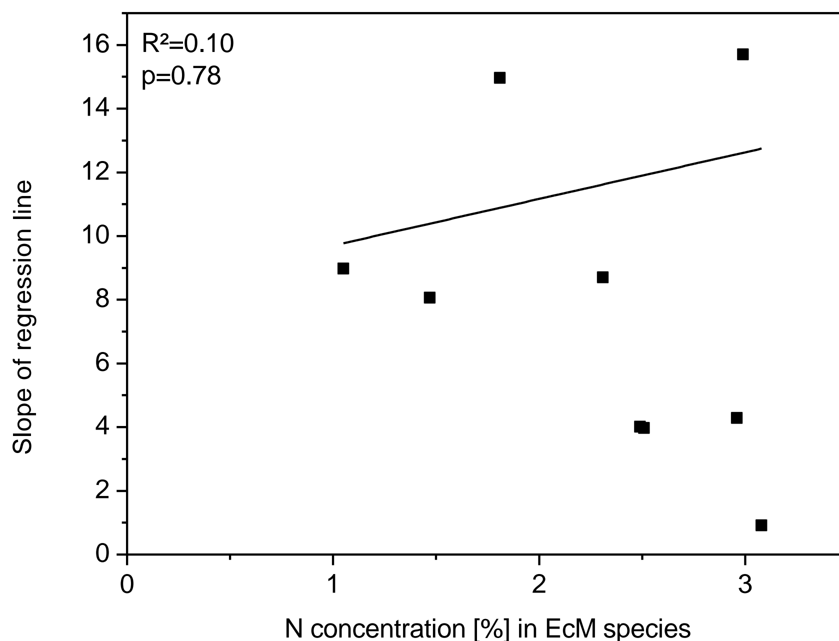


Fig. 4: Linear regression of N concentration [%] in EcM fungal species vs. slope of ^{15}N accumulation regression lines (confer Table 2).

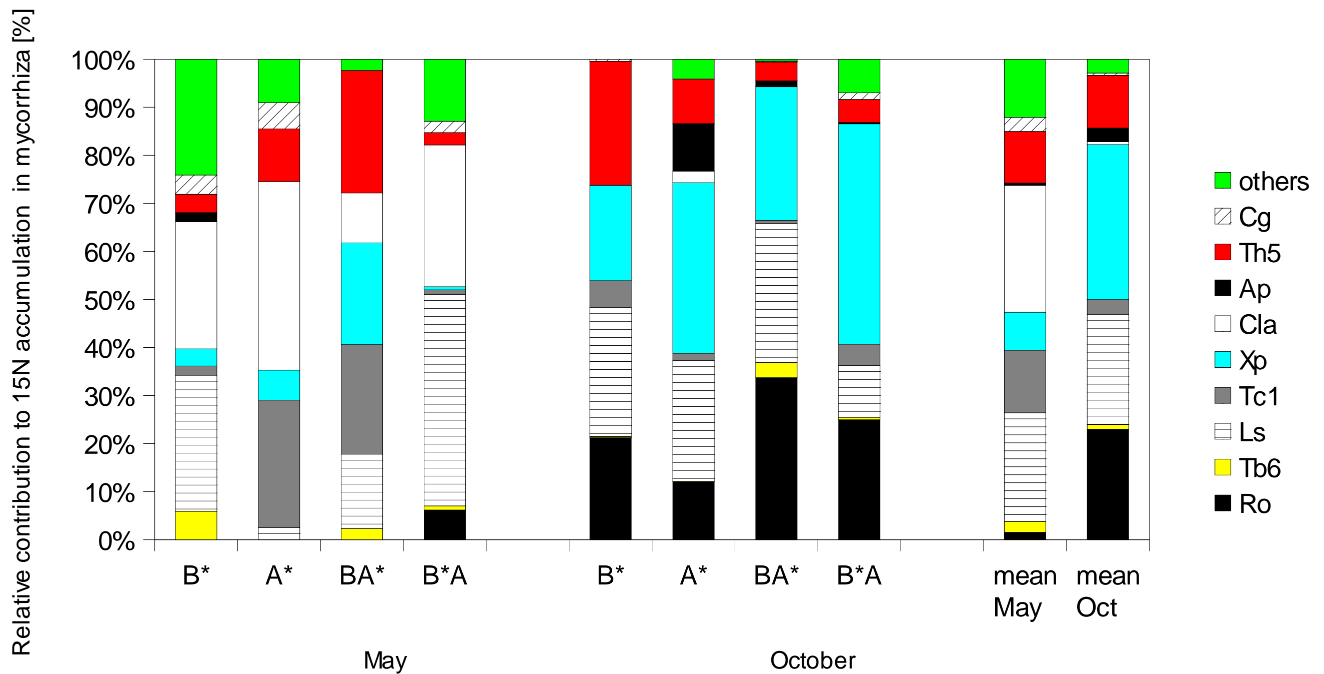


Fig. 5: Relative contribution of EcM fungal species to ^{15}N accumulation in mycorrhiza. B = Beech leaf litter, A = Ash leaf litter. Asterisks (*) indicate labelled leaf litter types. Ap= *Amanita rubescens*, Cla=*Clavulina* spec., Ls=*Lactarius subdulcis,* Ro=*Russula ochroleuca*, Tc1=*Tomentella castanea*, Tb6 = Tomentella badius, Th5=*Thelephora* spec., Xp=*Xerocomus pruinatus.* Others= species with low relative abundance. For relative abundance of EcM fungal species, see Supplementary Table S1. Values of relative contribution of EcM fungal species to ^{15}N accumulation in mycorrhiza are reported in Supplementary Table S3.

3.4.4 Root mass and accumulation of ^{15}N in roots

Higher N supply by N release from leaf litter could influence root biomass. However, correlation of total root dry mass with net N release [mg] from leaf litter revealed that root mass production increased not significantly after one vegetation period (p=0.06; Fig. 6).

The relationship between the amount of ^{15}N released from leaf litter vs. the amount of ^{15}N detected in root mass [µg APE] revealed a sigmoidal relationship (Fig. 7), with saturation of the curve on second harvest in October.

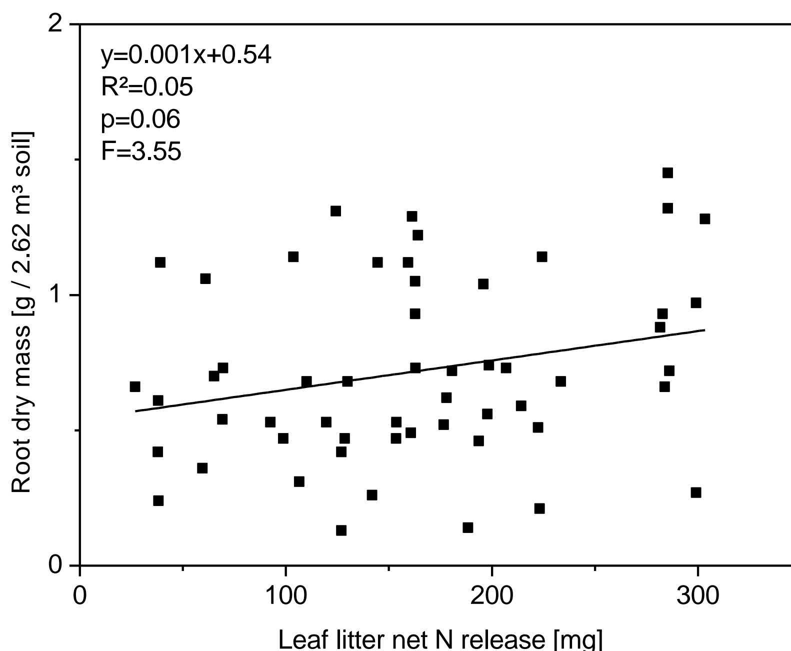
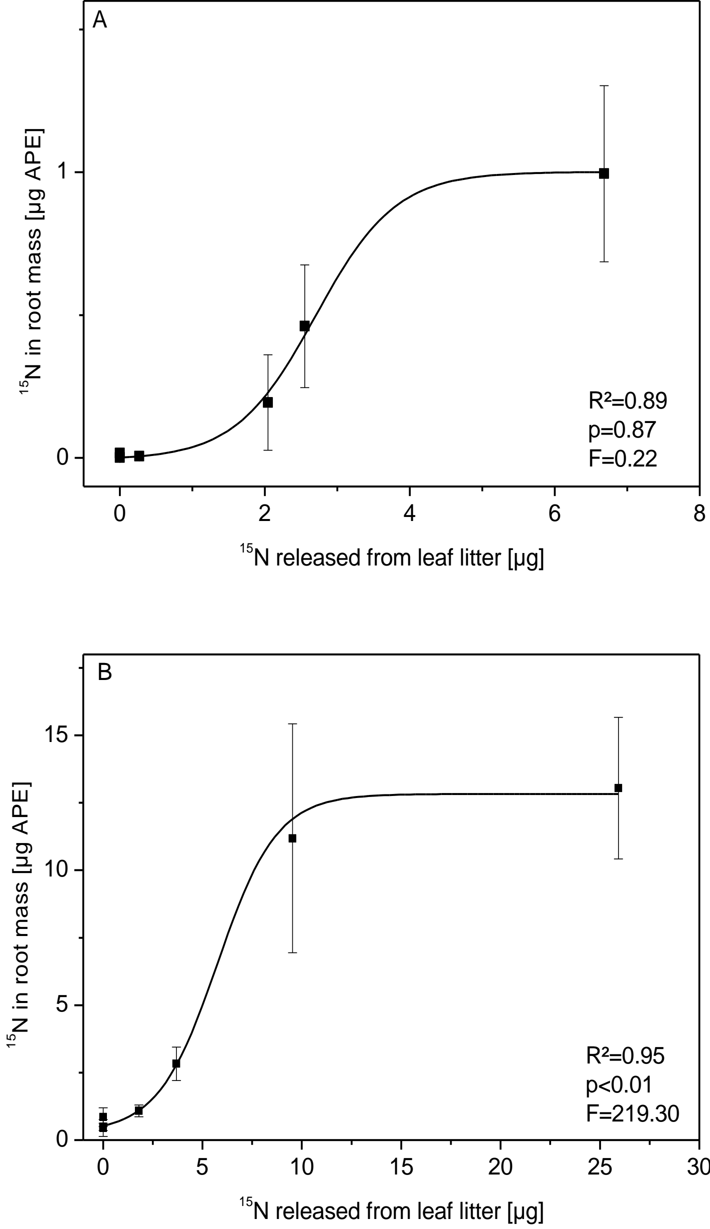


Fig. 6: Linear regression of root dry mass [g/2.65 m³ soil] vs. net release of nitrogen [mg] from leaf litter. Only data of second harvest in October were included in the analysis.

Fig. 7: Regression of ^{15}N in root mass [µg APE] vs. ^{15}N [µg]released from leaf litter. (A) May (B) October



3.5 Discussion

3.5.1 EcM fungal species on root tips differ in N concentration, which is not influenced by labelling or leaf litter species

This study shows that EcM fungal species colonizing root tips differ significantly in N concentration (Fig. 1). In our study, total N concentration of EcM species was not affected by a different leaf litter treatment, i.e. N concentration of the leaf litter species or labelling intensity. Increase in ^{15}N because of labelling did not influence N content of the fungi. This is consistent with Clinton et al. (1999) who assumed that differences in sporocarp nutrient concentrations among fungal species were related to species rather than related to substrate and with observations by Koide and Malcolm (2009), who determined N and C content and decomposition rates of several strains of ectomycorrhizal fungi. They found significant differences between the fungal strains, which were not affected by nutrient content of the growth medium. Similarily, Tedersoo et al. (2012) determined ^{15}N values on EcM root tips in a tropical forest and found that N concentration was not correlated with stable isotope patterns of EcM. N concentration of EcM species seems to be mainly determined by cell wall properties (Wallander et al. 2003). Fungi accumulate nutrients in their biomass. Differences in nutrient concentrations in fungal tissues are ecologically important since they serve as storage organ for nutrients which can either be released to the host plant, used by the fungus itself or serve after death and decay of the fungal tissues as food source for plants or other soil organisms.

3.5.2 Different EcM fungal species show different effectiveness for N assimilation mirrored by ^{15}N isotope signatures

In our study, roots and EcM fungi on root tips located 5-10 cm in the soil (mineral soil) received ^{15}N labelled compounds from the labelled leaf litter over a minimum distance of 5 cm. The ^{15}N content in the fungal tissues increased during the experiment. EcM fungi are usually located in the upper soil layers, where higher amounts of nutrients can be found compared to the mineral soil (Buée et al. 2007). There is evidence that EcM fungi use nitrogen and phosphorus directly from leaf litter (Bending and Read 1995; Perez-Moreno and Read 2000; Zeller et al. 2000; Pena et al. 2013). EcM fungi have the enzymes needed for the degradation of leaf litter, and although there are interspecific differences in enzyme activity

profiles of different EcM species (Diedhiou et al. 2010), they might have low ability to directly degrade nutrients from the leaf litter compared to saprotrophic fungi. Nonetheless, the EcM fungi in our study located in 5-10 cm soil depth received the labelled compounds from the leaf litter, either via leaching, as degradation products of other soil organisms, or via hyphal transport. Saprotrophic fungi in the leaf litter horizon degrade leaf litter substrates and make the C and N compounds available to other organisms; mycorrhizal fungi in the lower soil layers are able to take up N from the soil. (Lindahl et al. 2007; Hobbie et al. 2014). Subsequently, the assimilated N is stored in fungal tissues or transferred to the host plant.

We found significantly different accumulation rates of several EcM fungal species colonizing root tips in a natural forest. The EcM fungal species showed also differences in the relative contribution to ^{15}N accumulation in mycorrhizal fungi (Fig. 5). Using several N sources and two EcM and one ericoid mycorrhizal fungi on petri dishes in a laboratory experiment, Emmerton et al. (2001) demonstrated differences in N utilization between species and substrates. Lilleskov et al. (2002) analyzed different EcM fungal species in a laboratory study and showed differences in pure culture organic N use as well as ^{15}N natural abundance. ^{15}N in ECM fungi is generally highly variable intra and interspecific. The few studies about EcM fungi colonizing root tips showed differences between species (Haberer et al. 2007; Tedersoo et al. 2012; Pena et al. 2013).

Our data confirm the absence of a relationship between fungal N concentration and ^{15}N accumulation (Fig.4). Taylor et al. (2003) assumed that high ^{15}N values might be related to the high N % of these species. Hobbie et al. (2001) found a positive correlation between ^{15}N and N % for saprotrophic fungi, but not for mycorrhizal fungi. The same pattern was documented in a study by Hobbie et al. (2014), where genus significantly influenced ^{15}N in sporocarps of EcM and saprotrophic fungi, while N concentration affected ^{15}N in saprotrophic but not in EcM fungi. Analyzing ^{15}N in mycorrhizal root tips, Hobbie et al. (2008) found a correlation between ^{15}N and N % in an ammonium treatment, but not in a nitrate treatment. Other studies did not find a relationship between ^{15}N values and N concentration in EcM fungal root tips (Tedersoo et al. 2012; Pena et al. 2013).

It was supposed that differences in ^{15}N accumulation were related to differences in the length and exploration pattern of extramatrical hyphae produced by each fungus (Hobbie and Agerer 2010). In our study, the only species which belongs to Long distance (LD) exploration type is *Xerocomus pruinatus* (Xp), which showed comparably high, but not the highest accumulation

rate of ^{15}N. Since we sampled only one species of LD exploration type, this is not conclusive and is no sign of a generell pattern. Although we only obtained few samples of *Cenococcum geophilum* (n=2) in the unlabelled leaf litter treatment, this species showed high ^{15}N values (18 δ ‰, repectively), which is not in line with SD exploration type of *Cenococcum*, expecting low ^{15}N values. But this is in agreement with a study of Lilleskov et al. (2002), who found a single sclerotium of *Cenococcum* with high ^{15}N value of 15 δ ‰. Jones et al. (2009) injected ^{15}N labelled ammonium, aspartate and nitrate around seedlings colonized by *Cenococcum* and *Wilcoxina*, and found significant differences between these two fungal species in ^{15}N signatures, but both species belong to short distance (SD) exploration type where low ^{15}N values are expected. Tedersoo et al. (2012) analyzed stable isotope patterns in a tropical forest and found that ^{15}N values were determined by fungal lineage, but not by exploration type.

3.5.3 ^{15}N accumulation in roots and development of root mass after incraesed N release from leaf litter

^{15}N values in roots were lower compared to EcM fungi; this pattern has already been reported in many studies (Zeller et al. 2008; Högberg et al. 1996). Fungal sheaths stripped off from EcM of beech were 2.4 to 6.4 ‰ enriched in ^{15}N abundance relative to the root core. Total root dry mass did not significantly increase with increasing net N release from leaf litter after one vegetation period (Fig. 6). Since our study was a short term study about eleven months, we can not draw any conclusions about long term effects of increased N deposition. ^{15}N accumulation curve from labelled leaf litter showed a sigmoidal relationship (Fig.7), with saturation of the curve on second harvest in October. This might indicate transport processes since ^{15}N is either stored in EcM fungal root tips or transferred to the host plant (Pena et al. 2013). Leaching of N into lower soil layers might be a second explanation for this effect, but this possibility was neglected by Langenbruch et al. (2014).

3.5.4 Conclusion

In our experiment we analyzed ^{15}N isotope patterns on EcM root tips in a pure beech forest. Analysis of ^{15}N stable isotopes in EcM root tips is of great importance since different fungal structures like mycelium, fruitbodies (stipe and gills) and mycorrhizal root tips differ in their ^{15}N composition. Even different parts of fruitbodies (cap, gills and stipe) differ in ^{15}N (Zeller

et al. 2007; Hobbie and Högberg 2012). Additionally, ^{15}N stable isotope measurements in EcM fungal root tips allow the analysis of species without sexual reproduction and species that do not form fruitbodies (for example the ascomycete *Cenococcum geophilum*). We conclude that EcM species on root tips differ in ^{15}N accumulation rates and their relative contribution to total mycorrhizal ^{15}N accumulation. This might mirror functional differences between mycorrhizal species, substrate use, hyphal development, physiological status of the fungi combined with time and environmental variables. Stable isotope measurement of EcM fungal species colonizing root tips is possible and provides an important additional view on nutritional aspects of EcM fungi, which were fruitbody-focused for a long time.

3.6 Acknowledgements

We are grateful to the Deutsche Forschungsgemeinschaft (DFG) for financial support for the graduate school GRK 1086 "Die Bedeutung der Biodiversität für Stoffkreisläufe und biotische Interaktionen in temperaten Laubwäldern" (project A1). We thank the National Park administration for the permission to conduct our experiment in the area of the Hainich National Park. We thank Christina Langenbruch for providing leaf litter data, our colleagues of the graduate school and of the Department of Forest Botany for help with field sample collection and technical support and Thomas Klein for help with molecular work.

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Supplement: Tables S1-S3

Table S1: Mycorrhizal species found in the study. Abundances of EcM species under different leaf litter treatments. Nitrogen content and litter degradation of different leaf litter treatments. C = Contact exploration type, L = Long distance exploration type, M = medium distance exploration type, S = short distance exploration type, n/a = not applicable. B = beech litter forest, B* = Beech litter greenhouse, A = Ash litter forest, A*= Ash litter greenhouse, BA= mixed litter beech + ash forest, B*A= mixed litter beech greenhouse + ash forest, BA* = mixed litter beech forest + ash greenhouse.

Table S2: Supplementary Table S2: ^{15}N, ^{13}C, N % and C% in EcM fungal species and roots during the course of the experiment.

Table S3: Relative contribution of EcM fungal species to ^{15}N accumulation in mycorrhiza. B = Beech leaf litter, A = Ash leaf litter. Asterisks (*) indicate labelled leaf litter types. Ap= *Amanita rubescens*, Cla=*Clavulina* spec., Ls=*Lactarius subdulcis,* Ro=*Russula ochroleuca*, Tc1=*Tomentella castanea*, Tb6 = Tomentella badius, Th5=*Thelephora* spec., Xp=*Xerocomus pruinatus.* Others= species with low relative abundance

Supplementary table S1: Mycorrhizal species found in the study. Abundance of EcM species under different leaf litter treatments. Nitrogen content and litter degradation of different leaf litter treatments.

					Abundances of EcM species under different leaf litter treatments																
Full name	Accession number Genbank	Best BLAST hit (%) und accession number of hit	nucleotide fragment (R/L)	**Abbreviation**	**B**	**B***	**A**	**A***	**BA**	**B*A**	**BA***	**B**	**B***	**A**	**A***	**BA**	**B*A**	**BA***	**Exploration type**	**Reference for expl-type and N and mass**
Russula ochroleuca	KC952686	UDB000772 Russula ochroleuca (99 %)	679	Ro	0,0	0,0	0,2	0,0	0,0	6,9	0,0	9,8	13,6	15,5	6,9	21,4	30,0	30,5	C	Courty et al., 2008
Russula spec.	KC952679	UDB000901 Russula nigricans (97%)	691	Rs	0,0	0,0	0,0	0,0	4,0	0,0	0,2	0,0	0,0	0,0	0,0	0,0	1,8	0,0	C	Courty et al., 2008
Tomentella botryoides	KC952675	UDB000255 Tomentella botryoides (100 %)	583	Tb6	0,8	2,8	7,2	2,5	2,1	0,8	2,2	2,4	1,4	1,8	2,5	2,6	0,6	10,2	C	Agerer, 2001
Lactarius subdulcis	KC952680	UDB000048 Lactarius subdulcis (99%)	844	Ls	11,5	16,1	23,2	11,1	19,1	33,2	14,3	7,3	21,3	18,5	19,1	16,0	17,3	24,9	C	Agerer, 2001
Tomentella castanea	KC952674	UDB000120 Tomentella castanea (99%)	668	Tc1	19,7	31,9	13,9	21,3	23,9	15,9	30,9	7,8	7,3	10,5	3,7	9,2	8,9	1,6	C	Agerer, 2001
Xerocomus spec.	n/a	n/a	n/a	Xs	1,2	0,0	1,7	1,2	0,0	0,0	0,0	0,0	0,0	0,0	11,0	0,0	2,0	0,7	L	Agerer, 2001
Xerocomus pruinatus	KC952677	UDB000049 Xerocomus pruinatus (99%)	872	Xp	3,4	2,6	2,0	5,1	10,0	0,6	21,1	13,4	8,7	5,4	7,3	30,8	28,5	20,1	L	Agerer, 2001
Laccaria spec.	KC952691	UDB001495 Laccaria maritima (98%)	655	Ll	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,5	0,0	0,0	0,0	0,0	0,0	0,0	M	Courty et al., 2008
Amanita rubescens	n/a	n/a	n/a	Ap	0,0	1,4	0,0	0,0	0,0	0,0	0,0	0,0	0,0	2,2	11,0	7,8	0,7	5,5	M	Courty et al., 2008
Thelephora spec.	KC952710	FM995568.1 Uncultured Thelephora (99%)	857	Th5	29,6	7,6	7,8	7,3	12,2	2,8	8,4	33,6	44,0	33,0	30,5	8,4	2,4	4,5	M	Courty et al., 2008
uncultured basidiomycete (Bryssocorticium)	KC952692	UDB016321 Byssocorticium caeruleum (94 %)	950	Bry	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,6	0,0	0,0	0,0	0,0	6,2	0,0	S	Courty et al., 2008
uncultured Pezizales	KC952678	FN393151.1 uncultured Pezizaeae (99%)	690	P19	0,0	0,0	7,1	0,0	0,0	3,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	S	Courty et al., 2008
Genea hispidula	KC952681	UDB001408 Genea hispidula (99%)	1297	Gh	0,8	5,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	S	Agerer, 2001
Cenococcum geophilum	n/a	n/a	n/a	Cg	0,9	1,9	1,4	7,9	2,0	2,6	10,4	21,5	0,4	1,4	0,0	2,0	1,6	1,6	S	Courty et al., 2008
uncultured Pezizaeae	KC952688	FJ013079.1 uncultured ectomycorrhiza (Peziza) clone (100)	1284	P56	6,8	6,4	4,4	9,2	6,4	10,4	2,1	2,0	0,0	11,7	4,4	0,0	0,0	0,4	S	Courty et al., 2008
uncultured ECM (Clavulina)	KC952704	EU862208.1 Clavulina cf. Amethystina (99%)	1123	Cla	23,7	22,3	35,4	34,4	20,3	23,1	10,3	1,6	0,0	0,0	3,6	1,8	0,0	0,0	M	Courty et al., 2008
uncultured basidiomycete (	KC952707	GU327421.1 Uncultured Hydnotrya clone (95%)	1127	Ht	0,0	0,0	2,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	U	
Nitrogen content (mg per cylinder)				D N	-42	63	36	-11	-45	-22	-11	47	215	157	270	108	184	181		Langenbruch 2012
Litter degradation (% loss of litter mass)				D Mass	0,0	49,0	9,0	27,0	1,0	10,0	15,0	63,0	97,0	73,0	95,0	81,0	82,0	82,0		Langenbruch 2012

*Exploration type C = contact, M = medium rage, s = short distance, L = Long distance, u = unknown

Supplementary Table S2: ^{15}N, ^{13}C, N % and C% in EcM fungal species and roots during the course of the experiment. B = Beech leaf litter, A = Ash leaf litter. Asterisks (*) indicate labelled leaf litter types. Ap= *Amanita rubescens*, Cg = Cenococcum geophilum, Cla=*Clavulina spec*., Ls=*Lactarius subdulcis*, Roots= Fine roots, Ro=*Russula ochroleuca*, Tc1=*Tomentella castanea*, Tb6 = *Tomentella badius*, Th5=*Thelephora spec*., Xp=*Xerocomus pruinatus.* NA = not applicable. N= number of samples.

	N concentration [%]		C concentration [%]		Delta ^{13}C		Delta ‰ ^{15}N									
	all treatments		all treatments		all treatments		Unlabelled samples		B*		B*A		BA*		A*	
	may	oct	may	oct	may	oct	may	oct	may	oct	may	oct	may	oct	may	oct
Amanita (Ap)	2.64 n=1	1.81 ± 0.63 n=10	38.76	25.53 ± 10.23	-27.00 n=1	-27.08 ± 0.68	NA	6.18 ±1.97 n=4	8.73 n=1	29.43 ±6.25 n=2		19.56 n=1	NA	NA	NA	292.38 n=1
Cenococcum (Cg)	3.2§ ± 1.74 n=3	1.41 ± 0.12 n=3	41.74 ± 1.43	36.18 ±3.95	-28.07 ± 0.15	-28.39 ± 0.13	NA	18.88 ± 1.39 n=2	NA	NA	NA	NA	1.252 n=1	58.15 n=1	15.72 ±11.80 n=2	NA
Clavulina (Cla)	2.51 ± 0.79 n= 31	2.31 ± 1.01 n=6	32.85 ± 9.94	32.06 ±10.66	-27.16 ± 0.85	-27.60 ± 1.33	3.741 ± 2.01 n= 13	NA	8.82 ±2.60 n=3	NA	5.62 ±4.00 n=6	33.54 n=1	14.03 ±11.06 n=3	378.85 ±331.98 n=4	30.59 ±11.55 n=6	350.87 n=1
Lactarius (Ls)	3.08 ± 0.65 n= 27	2.99 ± 0.84 n=30	40.76 ± 3.90	40.52 ± 5.47	-27.06 ± 0.86	-27.84 ± 0.67	2.22 ± 3.23 n=11	6.12 ± 3.83 n= 11	11.38 ±6.67 n=4	39.70 ± 30.50 n=6	4.09 ±2.795 n=3	19.52 ±4.38 n=5	7.06 ±5.69 n=6	226.85 ±175.57 n=7	7.03 ±6.37 n=3	370.10 ±240.92 n=3
Roots	1.76 ± 0.30 n=47	1.55 ± 0.34 n=48	44.49 ±3.38	45.54 ± 3.66	-28.18 ± 0.96	-28.09 ± 0.77	1.46 ± 2.30 n= 17	7.04 ± 5.62 n=19	1.54 ±7.59 n=8	20.91 ±12.38 n=8	2.22 ±1.13 n=7	15.95 ±6.97 n=8	1.25 ±1.99 n=7	93.25 ±109.88 n=7	6.59 ±4.59 n=8	102.15 ±69.17 n=7
Russula (Ro)	2.83 ±0.09 n=2	2.28 ± 0.75 n=23	36.42 ±7.01	32.66 ± 8.13	-27.15 ± 0.34	-27.68 ± 0.72	- 0.205 n=1	7.20 ±4.08 n=9	NA	55.93 ±7.03 n=4	4.08 n=1	29.13 ±11.16 n=4	NA	460.22 ±145.66 n=3	NA	515.25 ±189.18 n=3
Thelephora (Th5)	1.47 ± 0.58 n=15	1.05 ±0.50 n=29	19.57 ± 9.02	15.20 ± 7.35	-27.31 ± 0.87	-27.26 ± 1.07	1.10 ± 1.82 n=8	14.15 ±4.77 n=9	11.69 ±8.06 n=3	41.94 ±12.63 n=7	NA	50.78 n=1	31.29 n=1	533.56 ±448.69 n=4	54.70 ±39.87 n=3	245.06 ±160.61 n=8
Tomentella 1 (Tc1)	2.49 ±0.65 n=28	2.96 ±0.66 n=21	36.89 ±7.67	40.25 ± 4.80	-27.47 ± 0.65	-27.34 ± 0.63	1.25 ± 2.36 n=8	7.65 ± 2.20 n=7	1.32 ±2.50 n=7	25.03 ±11.27 n=3	0.10 ±2.89 n=4	16.53 ±7.23 n=6	6.63 ±3.12 n=4	105.08 ±30.38 n=2	27.485 ±32.16 n=5	112.17 ±134.41 n=3
Tomentella 2 (Tb6)	2.72 ± 0.23 n=5	1.94 ± 0.69 n=11	43.13 ± 1.25	42.17 ± 4.20	-27.49 ± 1.50	-27.76 ± 0.88	0.54 ± 2.36 n=2	5.98 ± 3.00 n=6	NA	16.50 n=1	NA	NA	NA	98.35 ±103.89 n=2	1.83 n=1	42.26 ±11.52 n=2
Xerocomus (Xp)	3.03 ± 0.49 n=2	2.91 ± 0.63 n=25	37.62 ±1.54	35.04 ± 5.48	-27.11 ± 1.29	-27.22 ± 0.96	- 2.14 n=1	6.82 ±5.4 n=7	7.32 n=1	62.16 ± 23.46 n=3	NA	33.14 ±18.12 n=6	NA	337.40 ±154.90 n=4	NA	475.72 ± 295.45 n=3

Table S3: Relative contribution of EcM fungal species to ^{15}N accumulation in mycorrhiza. Confer Figure 5. B = Beech leaf litter, A = Ash leaf litter. Asterisks (*) indicate labelled leaf litter types. Ap= ***Amanita rubescens*****, Cla=*****Clavulina*** **spec., Ls=*****Lactarius subdulcis,*** **Ro=*****Russula ochroleuca*****, Tc1=*****Tomentella castanea*****, Tb6 = Tomentella badius, Th5=*****Thelephora*** **spec., Xp=*****Xerocomus pruinatus.*** **Others= species with low relative abundance**

	May				October					
	B*	A*	BA*	B*A	B*	A*	BA*	B*A	mean May	mean Oct
Ro	0,00	0,00	0,00	6,18	21,24	11,99	33,77	24,96	1,55	22,99
Tb6	5,92	0,00	2,30	0,83	0,24	0,13	3,07	0,53	2,26	0,99
Ls	28,31	2,54	15,50	44,04	26,83	25,15	28,96	10,78	22,60	22,93
Tc1	1,93	26,52	22,80	0,96	5,60	1,56	0,65	4,44	13,05	3,06
Xp	3,55	6,23	21,17	0,64	19,86	35,47	27,81	45,83	7,90	32,24
Cla	26,45	39,25	10,40	29,50	0,00	2,42	0,00	0,00	26,40	0,61
Ap	1,92	0,00	0,00	0,00	0,00	9,89	1,27	0,30	0,48	2,86
Th5	3,80	10,96	25,50	2,56	25,80	9,28	3,89	4,78	10,71	10,94
Cg	4,02	5,46	0,00	2,38	0,43	0,00	0,19	1,40	2,97	0,50

CHAPTER

4

Subcellular nutrient element localization in ecto- and arbuscular mycorrhizas of field-grown beech and ash trees

4.1 Abstract

Beech (*Fagus sylvatica* L.) and ash (*Fraxinus excelsior* L.) form two different mycorrhizal types; beech is colonized by EcM fungi and ash is colonized by AM fungi. Nutrient acquisition and element storage might differ between these two types of mycorrhiza or even between mycorrhizal species of the same type.

We analyzed the subcellular element distribution in roots of ash and beech and their associated mycorrhizal species (*Clavulina cristata*, *Cenococcum geophilum* and *Lactarius subdulcis* on beech amd *Glomus* sp. on ash) in a mixed deciduous forest using transmission electron microscopy (TEM) equipped with electron dispersive X-ray microanalysis (EDX).

High element concentrations were found on ash and were mainly located in the fungal tissues (AM; *Glomus* sp.) and not in the plant cells. Field grown *Glomus* sp. showed higher element concentrations compared to EcM fungal species, especially for K. The three EcM species of beech differed in their element concentration as well as in the subcellular distribution of elements. High calcium concentration was found in *Clavulina cristata*, and high sulphur concentration was found in *Cenococcum geophilum*. Differences between field grown mycorrhizal fungi regarding their functional roles for nutrient acquisition and storage are discussed.

4.2 Introduction

In Central Europe most dominant tree species like beech (*Fagus sylvatica* L.) are forming ectomycorrhizas (EcM) while many arbuscular mycorrhiza (AM)-forming woody species are understorey trees and shrubs (Harley and Harley 1987). One exception is the AM-associated Common European ash (*Fraxinus excelsior* L.), which can be a stand dominating tree. In mixed forests roots of ash trees contain higher concentrations of phosphorus (P), sulphur (S), potassium (K) and magnesium (Mg) than beech roots, whereas the concentrattions of calcium (Ca) is lower in ash than in beech roots (Lang and Polle 2011). Beech roots are associated with a large variety of different EcM species and ash with *Glomus* sp. (Lang et al. 2011). The fungal contributions to differences in nutrient elements in roots are unknown.

When comparing the two mycorrhizal types on host plants developing both arbuscular and ectomycorrhiza (for example *Eucalyptus* or *Quercus* trees), some authors showed that EcM are more effective for P uptake than AM (Jones et al. 1998; Egerton-Warburton and Allen 2001). Besides enhanced nutrient uptake and translocation, the nutrient storage capacity of mycorrhizal fungi is an important feature of this symbiosis (Kottke et al. 1998). 17 EcM species of *Picea abies* were analysed with inductively coupled plasma-atomic emission spectrometry (ICP-AES) and electron energy-loss spectroscopy (EELS) and were found to differ in element storage capacity, with *Xerocomus badius* being the most efficient in storage of nutrients (Kottke et al. 1998). Short term phosphorus uptake rates uptake rates differed between several EcM species (Colpaert et al. 1999).

Common and abundant species on beech in the Hainich forest are *Lactarius subdulcis*, *Cenococcum geophilum* and *Clavulina cristata* (Lang et al. 2011). *Lactarius subdulcis* (contact exploration type) has a hydrophilic hyphal mantle (Taylor and Alexander 2005). *Cenococcum geophilum* (short distance exploration type) is an ascomycete which is characterized by a shiny black mantle, containing melanin, a component protecting the fungus from decomposition (Fernandez et al. 2013). While *Lactarius* and *Cenococcum* are frequent in many forests, there are far less reports on *Clavulina cristata*, an EcM species of the medium distance exploration type (Courty et al. 2008). *Glomus* spec. was found to be the main AM fungus on ash trees in the Hainich (Lang et al. 2011). *Glomaceae* are fast and extensive colonizers compared to other AM fungi (Hart and Reader 2002). Length of AM hyphae in soil can range from 2-29 m g^{-1} soil, which is comparably short to the length of EcM hyphae (3-600 m g^{-1} soil; Leake et al. 2004). Also for AM, differences in hyphal

spread were found and hyphal spread of *Glomus* sp. was intermediate compared to *Scutellopora calospora* and *Acaulospora laevis* in a laboratory study (Jakobsen et al. 1992).

The nutrient distribution in EcM and AM in different tissues at the subcellular level has been analyzed by transmission electron microscopy (TEM) combined with energy-dispersive X-ray-microanalysis (EDX) measurements. With EDX a differentiation between the fungal and plant structures and a localization of the subcellular element amounts is possible (Scheidegger and Brunner 1998; Bücking et al. 2002). This method has mainly been used in culture studies with different external nutrient supply conditions to determine the subcellular element distribution between plant and fungus (Bücking and Heyser 2000a; 2000b). EDX studies were carried out under controlled conditions on *Picea* and *Pinus* using several different EcM species like *Pisolithus, Suillus, Xerocomus, Hebeloma, Amanita, Laccaria*, where differences between species were found (Bücking and Heyser 1999; 2000a; 2000b; Bücking et al. 1998; 2002). One of the few EDX studies in natural forests used EcM species to compare EDX with ICP analysis (Rumberger et al. 2005).

The objectives of this research were to study differences in the subcellular element distribution (Mg, P, K, Ca, S) of beech and ash roots and their associated mycorrhizas under natural conditions in an old growth forest. For this purpose, we analysed the localization of nutrients in root cells, different EcM species on beech (*Clavulina cristata, Lactarius subdulcis, Cenococcum geophilum*) and *Glomus* spec. on ash. We hypothesized that the main dominant mycorrhizal fungal species on beech differ in their functional roles regarding mineral nutrient acquisition and that AM fungi accumulate higher nutrient element concentrations than EcM. This might be the reason for the higher concentrations of K, P, S and Mg in ash roots compared to beech roots.

4.3 Materials and methods

4.3.1 Sampling and fungal material

Samples were collected in the Hainich National park (Thuringia, Germany) at the 6th of November 2008 in a forest near the Thiemsburg (51°06'N, 10° 31'E), with a basal area of *Fagus sylvatica* of 16.6 m² ha^{-1} and of *Fraxinus excelsior* of 12.8 m² ha^{-1} (Guckland et al. 2009; Talkner et al. 2010). The study area is characterized by a pH (H_2O) of 5.3; C 27.8 mg g^{-1} DM; N 2.0 mg g^{-1} DM and P 0.4 mg g^{-1} DM. The soil at this site is characterized as Luvisol with parent material of Triassic Limestone covered with Loess. The mean annual temperature ist 7.5 °C and the mean annual precipitation is 670 mm. Detailed information about the study area can be found in Mölder et al. (2006) and Leuschner et al. (2009). Nine soil cores containing roots of ash and beech were sampled with a soil corer (8x20 cm), stored in plastic bags and transported immediately into the laboratory and processed the same day. When it was not possible to free the roots from adhering soil via mechanical methods, samples were immediately washed carefully with as less water as possible. Roots of beech colonized by EcM fungi were observed under a dissecting microscope (Stemi SV 11, Zeiss, Jena, Germany) and three frequent EcM species (*Cenococcum geophilum*, *Lactarius subdulcis* and *Clavulina cristata*; Lang et al. 2011; online resource goe-fungi http://www.uni-goettingen.de/de/goe-fungi/92309.html; accession numbers EU346870; EU346875; EU816621) were collected by morphotyping. From ash roots, approximately 30 root tips were collected.

4.3.2 Preparation of root tips for TEM-EDX X-ray microanalysis

Fresh root tips of beech and ash were collected in a small mesh and rapidly frozen in a mixture of propane:isopentane (2:1) cooled in liquid nitrogen. The samples were freeze-dried at -45°C for 3 days and stored dry over silica gel until further processing. Freeze-dried root tips were vacuum-pressure infiltrated with diethyl-ether (Merck, Darmstadt, Germany), and embedded with stepwise increasing concentrations in styrene-methacrylate (Merck, Darmstadt, Germany; Fritz 1989). The samples were embedded in gelatine capsules in 100 % plastic. All steps of tissue processing were carried out under water-free conditions to prevent displacement or loss of diffusible elements in the root tissue. Polymerisation of the plastic-filled capsules took place in an oven at 60 °C over night and at 45°C for 10 days. After polymerization, semi-thin (1 µm) sections of the root tips embedded in plastic capsules were cut using a ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria) with dry glass knives. The sections were mounted with an adhesive decribed by Fritz (2007) on

hexagonal copper grids (Athene, provided by Plano, Wetzlar, Germany), coated with carbon and stored over silica gel.

4.3.3 Staining with Toluidine Blue and light microcscopy

For the detection of mycorrhizal structures, semi-thin sections of root tips were cut from the same blocks used for X-ray microanalysis, stained with Toluidine Blue (Merck, Darmstadt, Germany), mounted with Euparal (Carl Roth KG, Karlsruhe, Germany) on glass slides and visualised under the light microscope (Zeiss, Axioplan, Oberkochen, Germany). Sections were photographed with a digital camera (Zeiss, AxioCam MrC, Software AxioVision Release 4.6.3).

4.3.4 Scanning transmission electron microscopy (STEM) and X-ray microanalysis (EDX)

Semi-thin sections were analyzed by energy-dispersive X-ray microanalysis (EDX; EDAX DX-4, EDAX International Mahwah, NJ) under standardized conditions using a FEI Tecnai G² Spirit BioTWIN transmission electron microscope (TEM; FEI Company, Eindhoven, The Netherlands) as described by Chen et al. (2014). The microscope is equipped with a Si (Li) detector with a thin beryllium window (8 mm thick).

Point measurements as well as spectrum images (mappings) using the scanning transmission electron microscopy mode (STEM) in a matrix of 40x40 measurement points were conducted. The dwell time for each measurement point was 10.000 ms in the live second mode. The take-off angle was 15° tilt towards the detector. The accelerating voltage was 80 kV.

The spectra were analysed using the Tecnai Image Analysis (TIA)-Offline software (FEI Company, Eindhoven, The Netherlands). The spectra were automatically background fitted by the TIA-Offline software. Values of elements are given as background-fitted peak intensity. EDX spectra were collected between 0 and 10 keV. The peak centres of the different elements (K alpha) are sodium (Na) 1.04 keV, magnesium (Mg) 1.25 keV, aluminium (Al) 1.48 keV, silicium (Si) 1.74 keV, phosphorus (P) 2.01 keV, sulphur (S) 2.31 keV, chloride (Cl) 2.62 keV, potassium (K) 3.31 keV, calcium (Ca) 3.69 keV, iron (Fe) 6.38 keV and copper (Cu) 8.06 keV. The Cu measurement was overlayered by the Cu signal of the grids.

The relative element abundances in cross sections of plant roots and fungi were analyzed in cell walls and vacuoles of the following tissues: plant cell and plant cell-wall, for EcM fungi in the hyphal mantle and in the Hartig net, for AM in intercellular hyphae, intracellular hyphae and arbuscles. Ten replicates were investigated in each of these compartments in six different root

samples of each ash and beech (n=60). Data are means (+-SE). Statistical analysis of the data was performed using Analysis of Variance (ANOVA). Means were considered significantly different from each other, when the level of significance was $p \leq 0.05$.

4.4 Results

4.4.1 Differences in subcellular element distribution between fungal species and between compartments

For the analyis of the subcellular element distribution typical cross section of EcM and AM structures as depicted in Fig. 1 were chosen. Ectomycorrhizal roots on beech are characterized by a hyphal mantle (HM; Fig. 1 A) or hyphal sheath, where interwoven fungal hyphal cells surround the plant root. The Hartig Net (HN) surrounds the host plant cells. The arbuscular mycorrhiza (AM) of ash tree roots is characterized by arbuscles (Arb), inter- and intracellular hyphae (interH; intraH; Fig. 1 B).

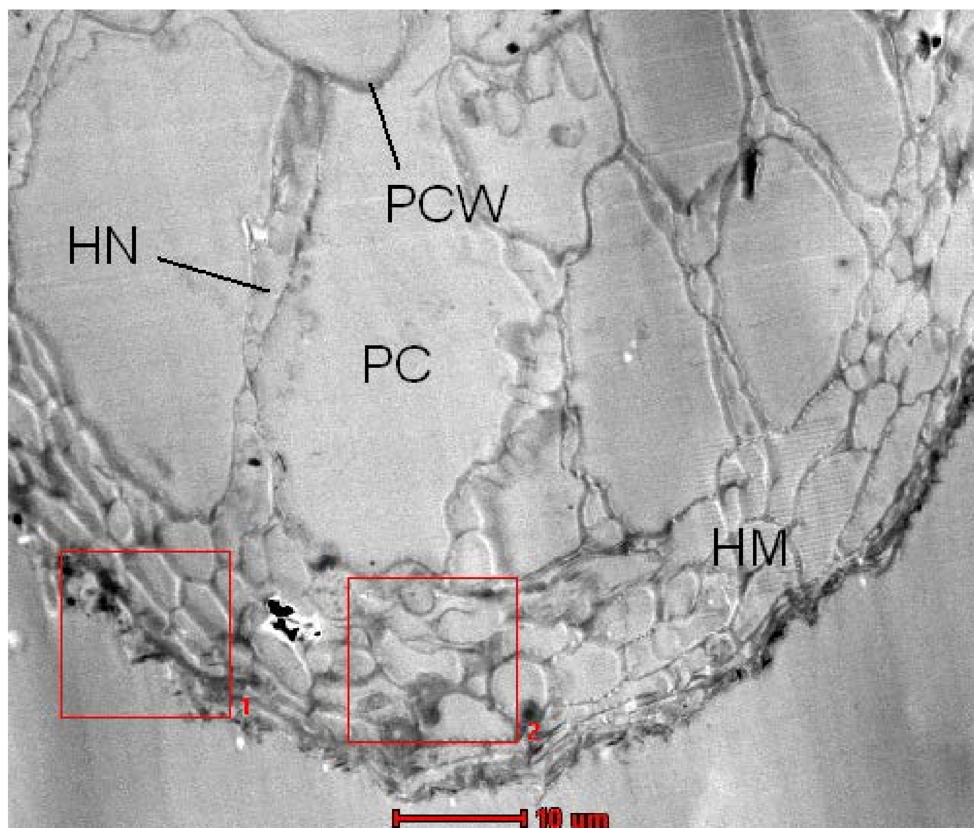


Fig. 1: Electron microscopic image of beech (A) and ash (B) roots and their associated mycorrhiza. (A): Beech (*Fagus sylvatica*) tree root colonized by ectomycorrhizal fungus *Lactarius subdulcis*. PCW = plant cell wall, HN = Hartig Net, PC = plant cell, FCW = fungal cell wall, HM = hyphal mantle. Bar = 10 µm. Red square = EDX-STEM mapping with 40x40 measurement points.

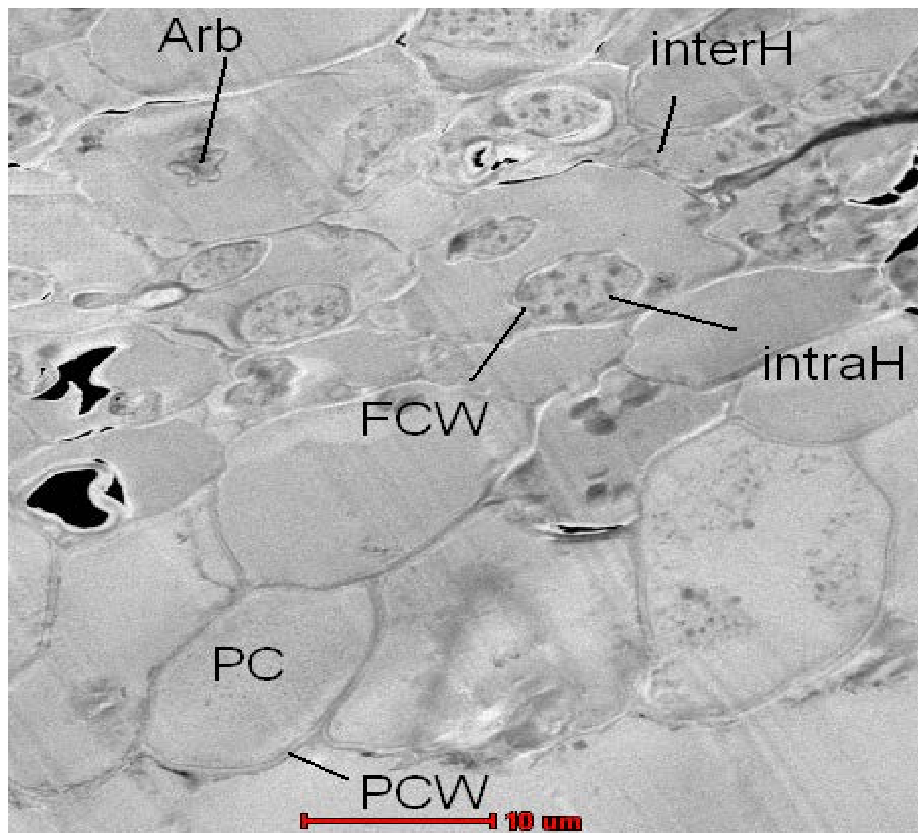


Fig. 1: Electron microscopic image of beech (A) and ash (B) roots and their associated mycorrhiza. (B): Ash (*Fraxinus excelsior*) tree root colonized by arbuscular mycorrhizal fungus *Glomus* sp.. Arb = arbuscles, FCW = fungal cell wall, intraH= intracellular hyphae, interH = intercellular hyphae, PC = plant cell, PCW = plant cell wall.

Figure 2 (EcM) and Figure 3 (AM) show the EDX-STEM-mappings for the major analysed elements. Subcellular element concentrations were not evenly distributed throughout the cell compartments, but show differences between plant and fungal compartments. P, K, S and Ca were mainly located around fungal cells of the HM (Fig. 2). In the intracellular hyphae (intraH) of *Glomus* spec. in ash roots, small granules were observed which contained mainly P, K and S (Fig. 3), while the hyphae were surrounded by a calcium containing periplasmatic membrane.

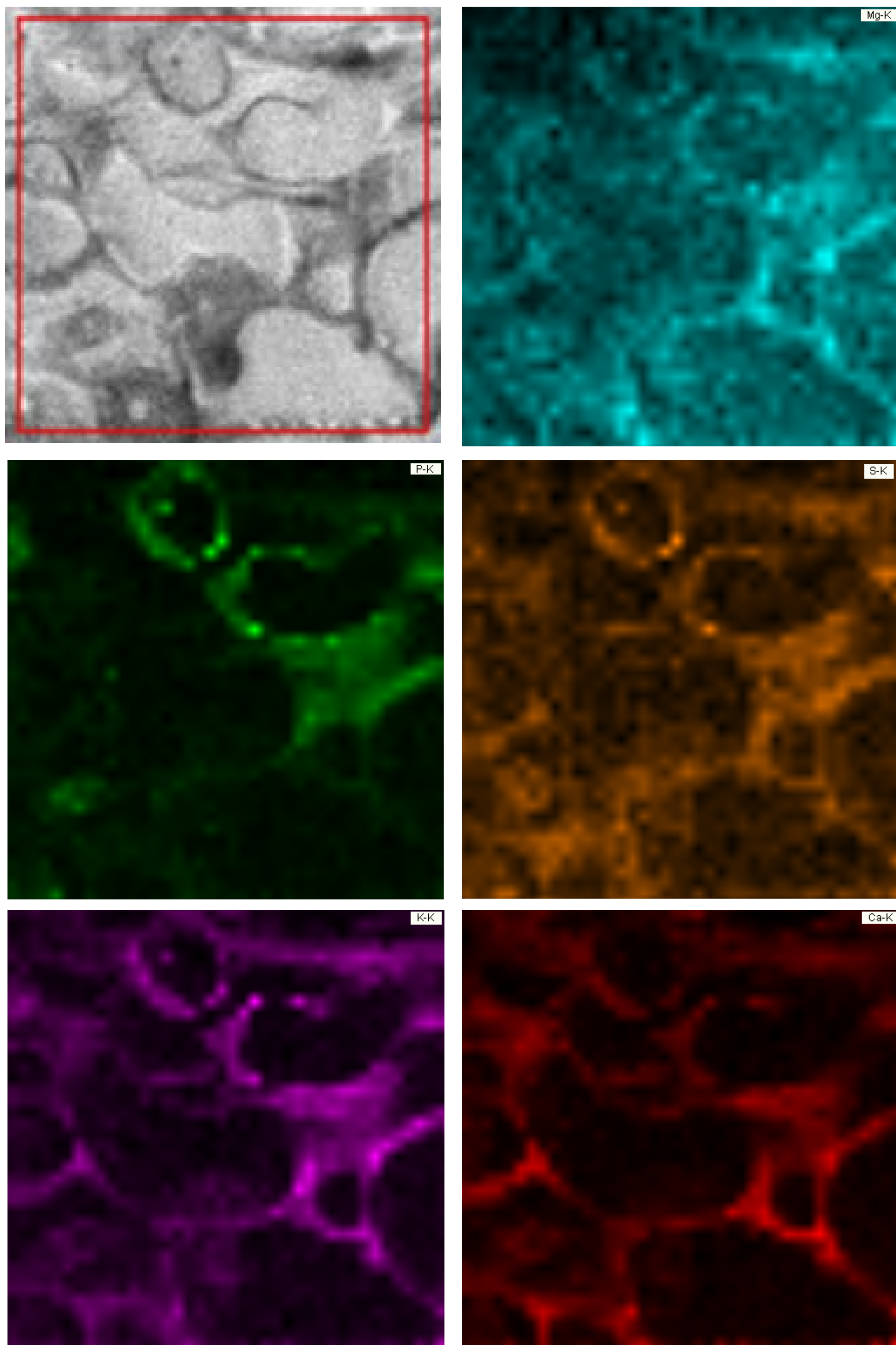


Fig. 2 A-F: EDX-STEM-mapping of ectomycorrhizal root of beech (*Fagus sylvatica*, colonized by *Lactarius subdulcis*) with 40x40 measurement points (A, red square). Element mappimgs of Magnesium (B, turquoise), Phophorus (C, green), Sulphur (D, orange), Potassium (E, purple) and Calcium (F, red).

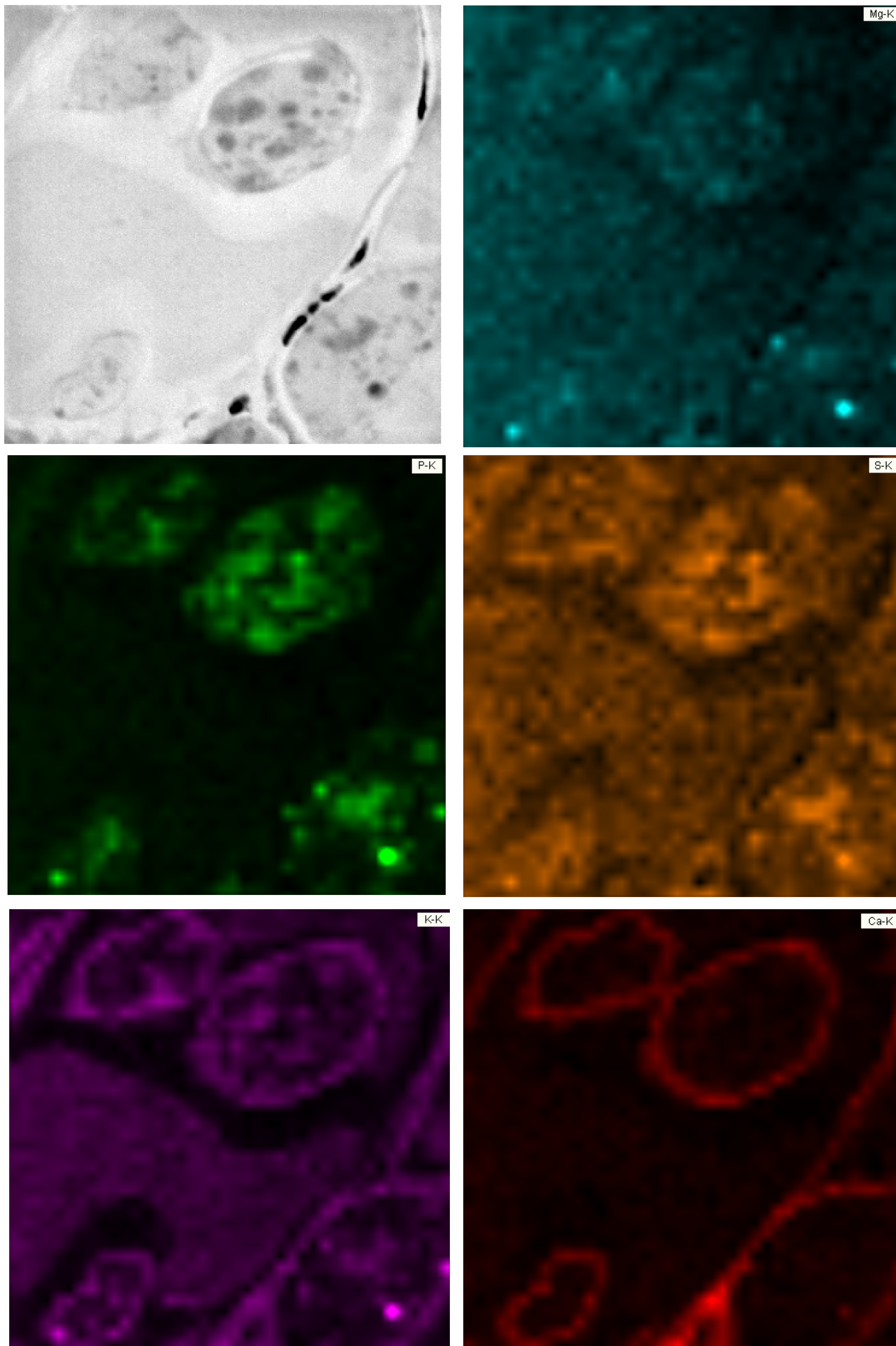


Fig. 3 A-F: EDX-STEM-mapping of arbuscular mycorrhizal root of ash (*Fraxinus excelsior*, colonized by *Glomus* sp.) with 40x40 measurement points (A). Element mappimgs of Magnesium (B, turquoise), Phophorus (C, green), Sulphur (D, orange), Potassium (E, purple) and Calcium (F, red).

A comparison of the relative element concentrations in the different mycorrhizal species revealed that *Glomus* spec. showed considerably higher element concentrations compared to the three EcM species, especially for K (Figure 4). We analyzed the element concentrations in the three EcM species (Fig. 4). *Cenococcum geophilum* showed higher sulphur concentration compared to *Clavulina cristata* and *Lactarius subdulcis* (Fig. 4), which were mainly located in the cell wall of the hyphal mantle (Fig. 5D). *Clavulina cristata* differed significantly in calcium concentration compared to the other two EcM fungal species, which were located in the cell walls of the HM and HN (Fig. 5E). For Mg, P and K, there was no significant difference between the EcM species (Fig. 4). In *Lactarius* and *Clavulina*, K and Ca differed significantly between the cells and the cell walls of the HM and HN (Fig.5 B,E). In *Cenococcum*, we found significant differences between the fungal compartments for Mg, S and Ca (Fig. 5 A,D,E). In *Glomus* spec. colonizing ash roots, K and Ca differend significantly between fungal compartments (Fig. 5 B,E).

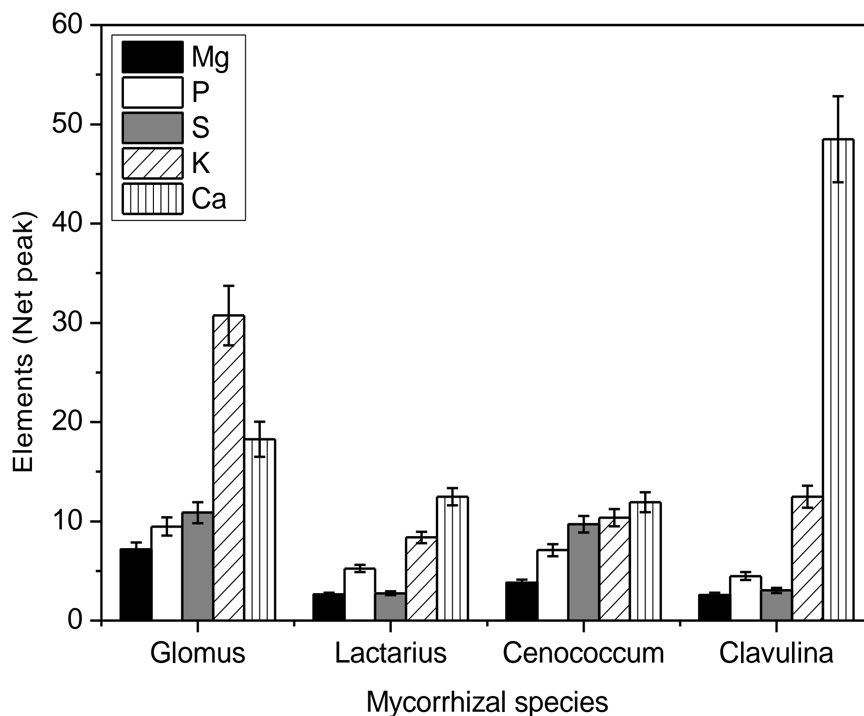
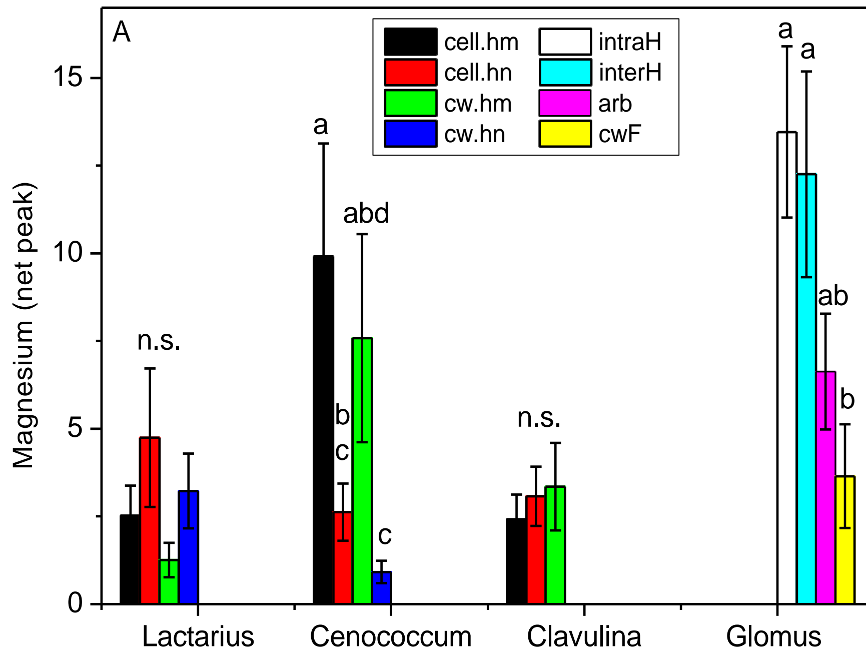


Fig. 4: Subcellular element concentration in mycorrhizal species. Mean values ± SE. Mg = Magnesium, P = Phosphorus, S = Sulphur, K = Potassium, Ca = Calcium.


A
cell.hm
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intraH
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cwF
Magnesium (net peak)
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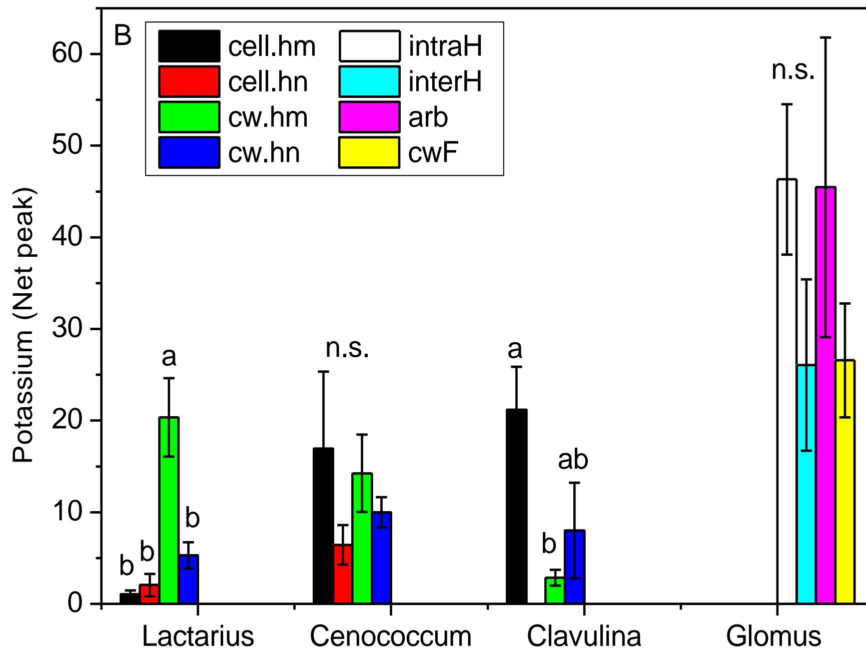
ab
b
Lactarius
Cenococcum
Clavulina
Glomus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Potassium (Net peak)
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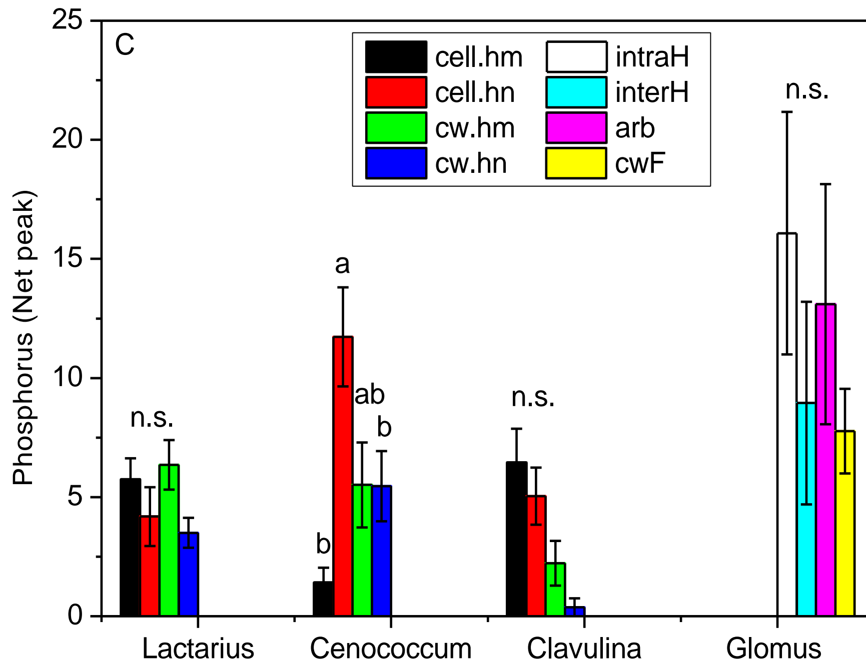
Lactarius
Cenococcum
Clavulina
Glomus
A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Phosphorus (Net peak)
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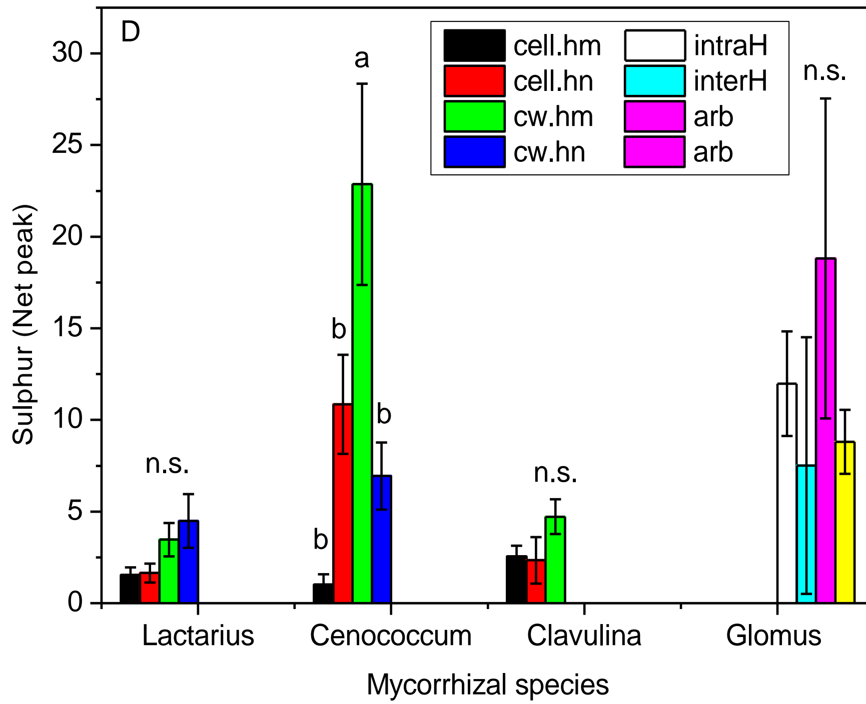
Cenococcum
Clavulina
Glomus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Sulphur (Net peak)
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Lactarius
Cenococcum
Clavulina
Glomus
Mycorrhizal species

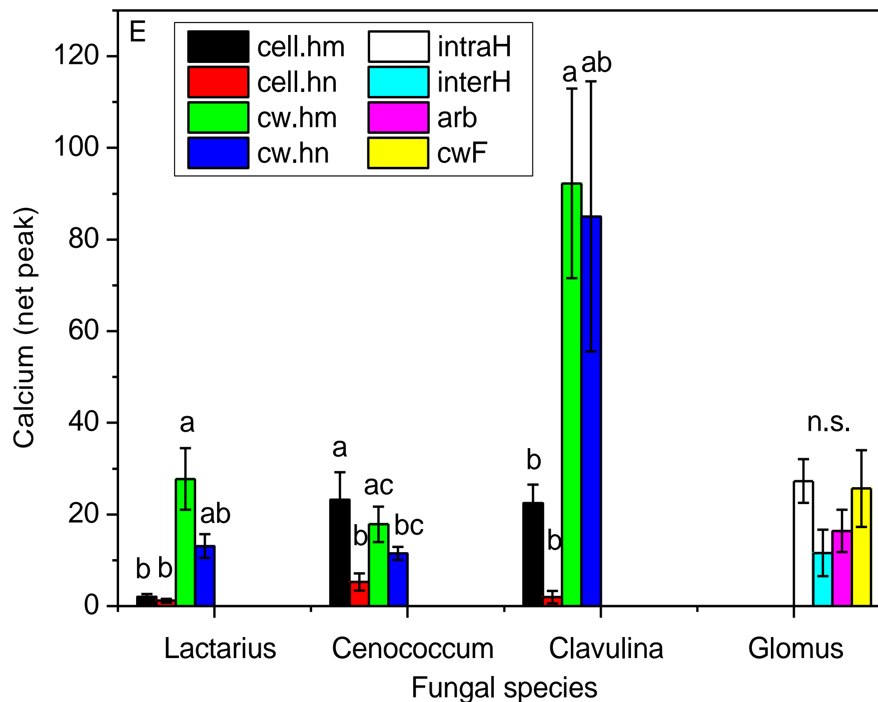

Fig. 5 A-E: Subcellular element amounts in different subcellular structures of mycorrhizal species. Mean values ± SE. Different letters indicate significant differences at p≤0.05. n.s. = not significant. (A) Magnesium, (B) Potassium, (C) Phosphorus, (D) Sulphur, (E) Calcium.

4.4.2 Nutrient concentrations are higher in fungal tissues compared to plant tissues

To investigate whether nutrients were higher in the fungal cells compared to the plant cells, the subcellular element concentration in roots of ash and beech trees and their associated mycorrhizal types were analysed (Fig 6 A, B). AM fungi contained significantly higher concentration of Mg, P, S and K than EcM (Fig. 6 B). Ash roots contained higher Mg and K concentrations than beech roots, but the P, S and Ca concentrations were not significantly different (Fig. 6 A).

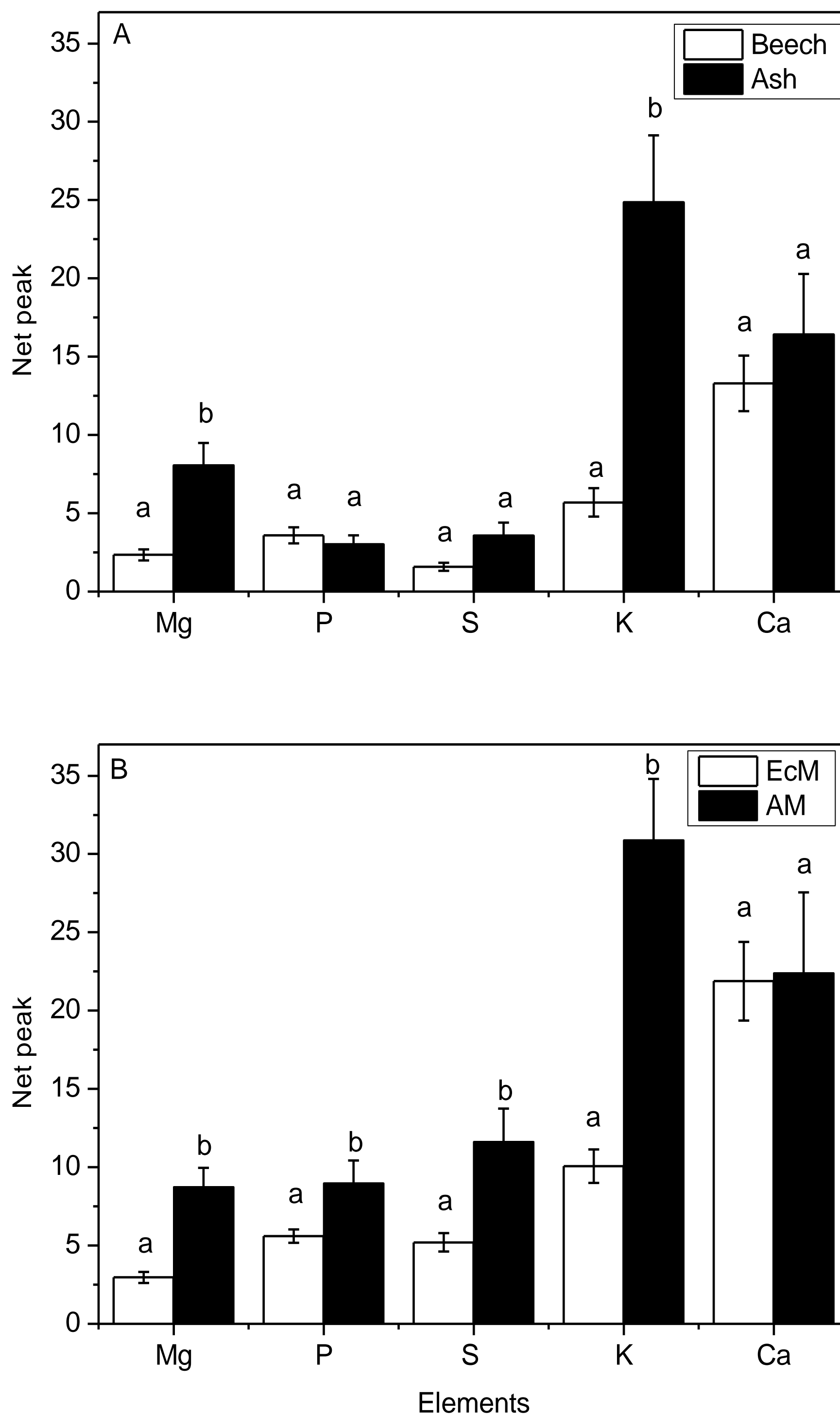

Fig. 6 A, B: Subcellular element concentration of Magnesium (Mg), Phosphorus (P), Sulphur (S), Potassium (K) and Calcium (Ca) in plant (A; beech, ash) and fungal (B; EcM, AM) structures. Different letters indicate significant differences at p≤0.05. Other elements at detection limit are shown in Table S1 (Supplement)

The subcellular element concentrations in fungi and the subcellular element concentrations in their host plant tissues were correlated in *Lactarius*, *Clavulina* and *Glomus* (Fig. 7), higher element concentrations in fungi were related to high element concentrations in plant tissues. However, this relationship was not existant in *Cenococcum* (Fig. 7; Tab. 1; R^2=0.06, p= 0.34).

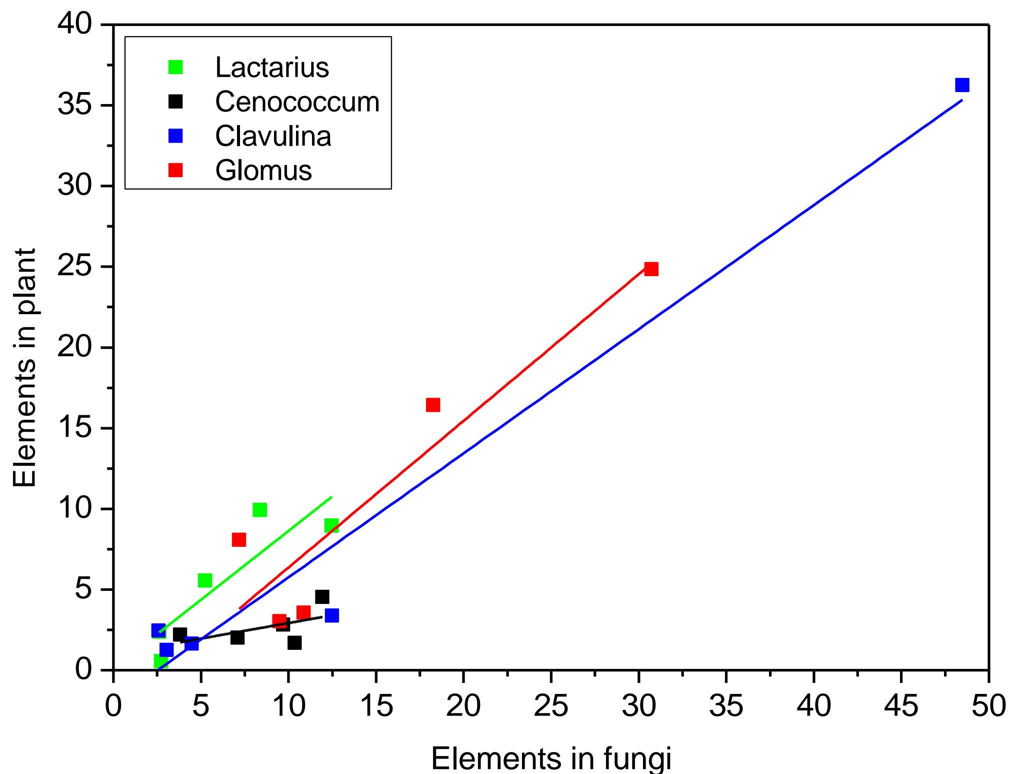

Fig. 7: Relationship between subcellular element concentrations in plant and fungal tissues of mycorrhizal species.

Tab. 1: Parameters of regression lines (Fig.7) of subcellular element concentration in plant tissues vs. subcellular element concentration in fungal tissues in the analyzed mycorrhizal species. $p < 0.05$ is marked in bold letters.

	Lactarius	*Cenococcum*	*Glomus*	*Clavulina*
Intercept	0.098	1.54	-2.72	-1.935
Slope	0.85	0.17	0.91	0.77
R^2	0.70	0.06	0.82	0.96
F	10.21	1.28	19.81	105.86
p	**0.049**	0.34	**0.02**	**0.00196**

4.5 Discussion

This study shows that the nutrient concentrations differed in AM and EcM of field-grown ash and beech. The peak intensities were higher in ash compared to beech and located in the fungal tissues and not in the plant. This is a good evidence of the fungal symbiont as accumulator of nutrients (Kottke et al. 1998). The high nutrient concentrations in AM could be an explaination for the high P, K and Mg concentrations of ash roots compared to beech roots (Lang and Polle 2011). The proportion of the area of the AM fungus in ash plant tissues was approximately 25 to 30 % (Fig. 1B), so it has to be questioned if the fungus is able with this relatively small proportion to significantly change the nutrient concentration of the whole ash root. But it is known that the fast degrading arbuscles of AM symbiosis have a lifetime of around 10 days (Sanders et al. 1977), and after that, the arbuscles collapse and release the stored elements into the plant tissues. Therefore, a fast nutrient transfer between plant and fungus leads to a constant support of nutrients to the host tree, which could result in high nutrient concentration in ash roots.

To our knowledge, this is the first EDX study about mycorrhizal root tips colonized by *Cenococcum*, *Clavulina* and *Glomus* in a natural forest. *Glomus* and the three EcM differed in their element concentration as well as the distribution patterns of the analysed elements and *Glomus* had the highest K, Mg and P concentrations (see above). The intracellular hyphae of *Glomus* sp. were surrounded by Ca (Fig. 3), which could indicate the cell walls of plant and fungus or the for AM typical periplasmatic membrane (interface compartment), an apoplastic compartment between the contact zone of plant and fungus (Bonfante-Fasolo and Scannerini 2004). This compartment consists of the membranes of both symbiontic partners, and contains pectin (Bonfante and Perotto 1995), which leads to the Ca signal. High Ca concentrations were also found in *Clavulina*. Beech roots were found to have higher Ca concentrations compared to ash roots (Lang and Polle 2011); a high proportion of *Clavulina* or similar EcM species with high Ca concentration could be responsible for that. We found no comparable study about nutrient concentration in *Clavulina*, but Hagerberg et al. (2005) showed high accumulation of Ca in the mycelium of *Piloderma* sp. and concluded that this fungus stores Ca in the cell walls of hyphae and the fungal mantle as calcium oxalate crystals, which increases hydrophobicity and serves as protection against grazing microbes. *Cenococcum* showed the highest S concentrations, which were mainly located in the cellwalls of the hyphal mantle. This fungus is known to have cell walls with a high melanin content, which is a sulphur-containing polymer. This high melanin content results in high resistence to decomposition (Fernandez et al. 2013) and might also serve as a protection against grazing animals.

Wallander et al. (2003) observed differences in K and Ca in *Suillus luteus* and *Paxillus involutus* and assumed species-related differences in the ability to accumulate nutrients. Rumberger et al. (2005) compared 12 EcM species in a beech-pine forest via EDX and ICP and found *Genea* and *Xerocomus* with higher elements compared to *Russula* and *Lactarius.* Since mycorrhizal fungal species differ in their anatomy, mantle structures and mantle properties, and the distribution of hyphae in soil (Agerer 1987-2008; Colpaert et al. 1992; Wang et al. 2011), it seems reasonable that these species are functional different regarding nutrient uptake, translocation and storage capacities. The accumulation of nutrients in mycorrhizal tissues can have several possible functions. The first function is the direct nutrient supply to the host. Second, the fungus stores nutrients to balance soil nutrient fluctuations when nutrients are present in high amounts. Third, fungi are known to form crystals to dectoxificate heavy metal contamination (Turnau et al. 1993).

Lactarius subdulcis is a generalist species with a broad distribution and showed in our study intermediate element amounts. This is in line with Rumberger et al. (2005) and Kottke et al. (1998) who also found *Lactarius* to be intermediate in nutrient concentrations compared to other EcM species and characterizes *Lactarius* as an established fungus, which is widely distributed in the forest area with a constant abundance and supplies the host tree constantly with nutrients. Kottke et al. (1998) showed that *Xerocomus* has high element storage capacity, with *Lactarius* intermediate and *Cenococcum* showing rather low element amounts compared to the other EcM species. *Cenococcum* has little host specifity, global distribution and is frequently abundant in EcM communities across all soil layers (Dickie 2007) and was declarated as an ultra-generalist fungus (Dickie and Reich 2005).

EcM and AM fungi differ fundamentally in the anatomical structure of the interface, specialized cells for the exchange of nutrients in mycorrhizae (Bonfante-Fasolo and Scannerini 2004). Both mycorrhizal types aim to increase their nutrient absorbing surface through branching of hyphae; either in the Hartig Net (EcM) or in the hyphae inside the roots in AM. In EcM, P was mainly located in the fungal hyphal mantle (HM), which completely surrounds the roots of many EcM trees and isolates the root from the surrounding soil. Nutrients must pass through that mantle, are stored in the fungal mantle or are translocated via the Hartig Net to the plant. Early publications by Harley and Mc Cready (1951) demonstrated that approximately 90 % of the accumulated P is located in the fungal sheath and not in the host tissues. Our study found approximately 2-fold higher P in fungal tissues compared to plant tissues (Fig. 6 and Supplementary Table S1). Ling-Lee et al. (1975) showed that trees store phosphate as polyphosphate granules in their mycorrhizas. They described

small granules in the sheath and Hartig net of ectomycorrhizas of *Eucalyptus* plants, in *Pinus* (EcM), *Arbutus* (ectendomycorrhiza) and hyphae and vesicles of *Liquidambar* plants (AM). Here we probably found these granules in the intracellular hyphae of *Glomus* sp. (Fig. 1B and Fig. 3). Cairney et al. (1988) showed that polyphosphate granules are also present in saprotrophic fungi. These granules are often also associated with K and Ca and are supposed to regulate the exchange between plant and fungus.

We showed the subcellular localization of P and other elements in roots of ash and beech. This is only a snapshot of the nutrient storage of these species in an undisturbed deciduous old growth forest in autumn, when stored nutrient concentrations are high (Lussenhop and Fogel 1999; Genet et al. 2000). Furthermore, it does not mean that the fungus provides all the stored elements to the host plant since fungi need some part of the stored nutrients for their own growth. EDX is a helpful method to determine the subcellular element distribution in plants and fungi in an old-growth forest under natural conditions.

4.6 Acknowledgements

This work was done in the framework of the graduate school GRK 1086 “Die Bedeutung der Biodiversität für Stoffkreisläufe und biotische Interaktionen in temperaten Laubwäldern” (project A1). We thank the National Park administration for the permission to conduct our experiment in the area of the Hainich National Park. We thank our colleagues of the graduate school and of the Department of Forest Botany for help with field sample collection and technical support and Dr. Andrea Olbrich for introduction into transmission electron microscopy.

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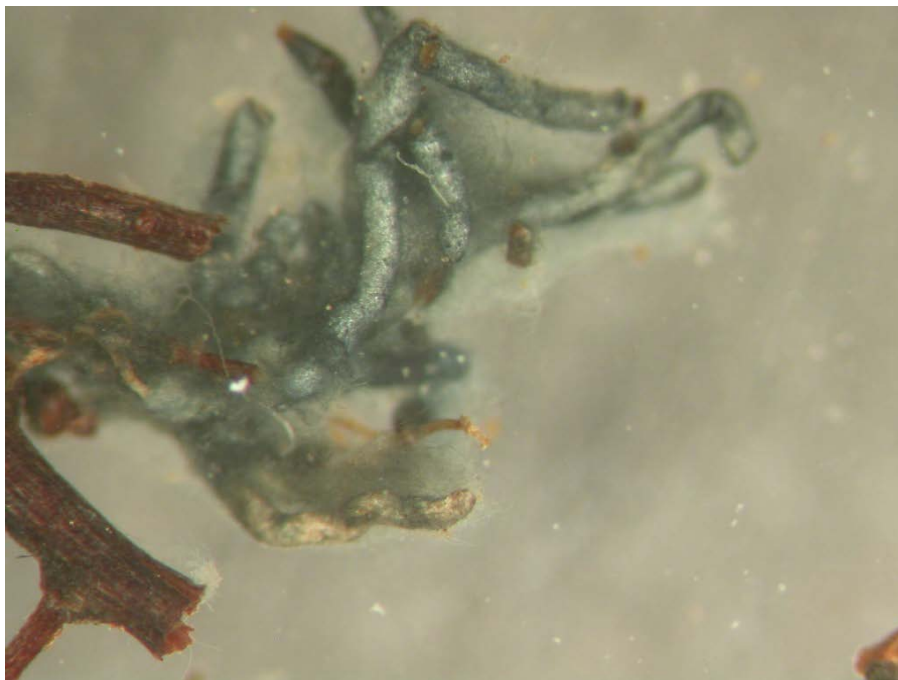
Supplementary Table S1: Elements below detection limit. Mean of net peak area ± SE. Na-K = Sodium, Mg-K = Magnesium, Al-K = Aluminium, Si-K = Silicium, P-K= Phosphorus, S-K = Sulphur, Cl-K = Chloride, K-K = Potassium, Ca-K =Calcium, Fe-K = Iron.

	Na-K	**Mg-K**	**Al-K**	**Si-K**	**P-K**	**S-K**	**Cl-K**	**K-K**	**Ca-K**	**Fe-K**
plant	7.12 ±1.16	4.05 ±0.51	10.34 ±1.35	11.86 ±1.04	3.42 ±0.39	2.07 ±0.28	7.59 ±0.84	11.42 ±1.48	14.23 ±1.69	3.01 ±0.57
fungus	6.83 ±0.95	4.10 ±0.38	24.70 ±3.98	11.45 ±0.91	6.26 ±0.45	6.45 ±0.64	7.54 ±0.69	14.16 ±1.21	21.99 ±2.25	8.53 ±1.56
ash	3.69 ±0.66	8.06 ±1.43	0.00 ±0.00	15.14 ±2.73	3.02 ±0.56	3.57 ±0.83	10.41 ±1.95	24.85 ±4.28	16.42 ±3.86	0.00 ±0.00
beech	8.58 ±1.62	2.34 ±0.36	10.34 ±1.35	10.47 ±0.91	3.59 ±0.52	1.58 ±0.26	6.39 ±0.85	5.69 ±0.91	13.30 ±1.77	3.01 ±0.57
fungus EcM	7.15 ±1.15	2.96 ±0.35	24.70 ±3.99	11.75 ±1.05	5.59 ±0.43	5.20 ±0.59	5.98 ±0.56	10.06 ±1.07	21.89 ±2.51	8.53 ±1.56
fungus AM	5.53 ±1.27	8.75 ±1.22	NA ±0.00	10.20 ±1.45	8.97 ±1.46	11.62 ±2.13	13.90 ±2.54	30.89 ±3.90	22.40 ±5.14	NA ±0.00
Lactarius	7.79 ±2.86	3.40 ±0.70	43.72 ±7.72	11.09 ±1.28	4.44 ±0.61	2.37 ±0.51	3.19 ±0.71	4.94 ±0.86	14.42 ±3.41	0.00 ±0.00
Cenococcum	10.71 ±2.36	3.81 ±0.75	22.96 ±7.04	15.25 ±1.62	7.09 ±0.99	9.70 ±1.48	8.69 ±1.07	10.36 ±1.70	11.93 ±1.43	13.16 ±2.36
Clavulina	1.68 ±0.42	2.58 ±0.55	13.19 ±2.51	9.69 ±3.06	4.48 ±0.82	3.04 ±0.57	4.98 ±1.05	12.48 ±2.60	48.49 ±8.00	0.46 ±0.33
Glomus	5.53 ±1.27	8.75 ±1.22	0.00 ±0.00	10.20 ±1.45	8.97 ±1.46	11.62 ±2.13	13.90 ±2.54	30.89 ±3.90	22.40 ±5.14	0.00 ±0.00

CHAPTER

5

Short communication: Presence of a competing tree species influences subcellular element localization in plant roots of Common European ash (*Fraxinus excelsior* L.) and European beech (*Fagus sylvatica* L.) and their associated mycorrhiza



5.1 Introduction

Mycorrhizal fungi are important components of forest ecosystems; plants deliver carbon to the fungus in return for phosphorus (P), nitrogen (N) and other mineral nutrients from the fungus (Smith and Read 2008). Mycorrhizal fungi take up nutrients from the soil, transfer them to the host plant if needed or accumulate them in their tissues. This storage function depends mainly on soil properties and plant host demand and shows a seasonal variation with peak a in autum (Genet et al. 2000).

Beech (*Fagus sylvatica* L.) is a stand dominating tree species which forms ectomycorrhizal symbiosis (EcM) while ash (*Fraxinus excelsior* L.) trees form arbuscular mycorrhiza (Read, 1991). In a previous study in the Hainich forest (Lang and Polle 2011), ICP-nutrient analysis revealed that the roots of ash trees have higher nutrient concentrations than beech roots, especially for the nutrients phosphorus (P), nitrogen (N), sulphur (S), potassium (K) and magnesium (Mg). Calcium (Ca) concentrations were lower in ash than in beech roots. Additionally, it was found that an increase of tree species diversity also leads to changes in root nutrient concentration of ash and beech. Possibly competitive effects between the different tree species were the reason for differences in nutrient concentrations (Lang and Polle 2011).

One possibility to determine the nutrient storage capacity of the fungus and the plant is scanning transmission electron microscopy (STEM) micromapping combined with electron dispersive x-ray-microanalysis (EDX) measurements. With this method a differentiation between the fungal and plant structures and a localization of the subcellular elements is possible.

The objectives of this research were to investigate if element amounts in plant and fungal structures of ash and beech roots are influenced by the presence of a con- or heterospecific neighbouring tree. We hypothesize that the presence of a neighbouring tree (pure tree species or a mixed group of beech and ash trees) influences the nutrient concentrations in the different subcellular tissues of plants and fungi. (1) In mixed treatments, in presence of a heterospecific neighbouring tree species, nutrients are higher because of complementary resource-use. (2) In pure treatments, in presence of a conspecific neighbouring tree species, nutrient concentrations are lower because of higher competition between trees.

5.2 Materials and methods

5.2.1 Study area

The study took place in the Hainich National park, which is an oldgrowth broadleaved forest in Thuringia, Germany. This forest is unmanaged since at least 1935, where it first became a military training area and since 1997 a national park. The mean annual temperature ist 7.5 °C and the mean annual precipitation is 670 mm. The soil of this site is characterized as Luvisol; with parent material of Triassic Limestone covered with Loess. Detailed information about this forest and the experimental plots can be found in Mölder et al. (2006) and Leuschner et al. (2009). The study took place in the plot DL 3c; stand and soil characteristics and a map of the exact location of this study plot can be found in Guckland et al. (2009).

5.2.2 Sampling of fungal material

Sampling took place at the 6th of november 2008 in the study area DL 3c near the Thiemsburg (51°06'N, 10° 31'E) in the Hainich national park, where either small groups of only ash or beech trees (mono-treatment; minimum of four trees per species) or a mixed group of both tree species (mixed treatment) occur. Soil samples including roots of ash and beech were taken with a soil corer (8x20 cm) and stored in plastic bags. There were three replications: three soil cores in a pure beech treatment, three soil cores in a pure ash treatment and three soil cores in a mixed treatment of beech and ash.

Field-collected roots were brought immediately into the laboratory and processed the same day. When it was not possible to free the roots from adhering soil via mechanical methods, samples were immediately washed carefully with as little water as possible, viewed under a dissecting microscope and sorted according to the different morphotypes. Easily recognizable and most frequent EcM species in the Hainch forest are *Cenococcum geophilum*, *Lactarius subdulcis* and *Clavulina cristata* (Lang et al. 2011 and online resource goe-fungi http://www.uni-goettingen.de/de/goe-fungi/92389.html).

From ash samples, several root tips were cut. It was shown in a previous study (Weiße 2008) that the AM colonization level on ash in the Hainich is very high and that there is a high probability of getting mycorrhizal root tips. Approximately 30 root tips (<1cm long) were cut out of every of the four treatments.

5.2.3 Preparation of root tips for TEM-EDX analysis - Sample preparation and X-ray microanalysis

Fresh root tips of beech and ash roots were collected in a small mesh and rapidly frozen in a mixture of propane:isopentane (2:1) cooled in liquid nitrogen to -196°C. The samples were freeze-dried at -45°C for 3 days and stored dry over silica gel until further processing. Freeze-dried root tips were vacuum-pressure infiltrated with diethyl-ether, and embedded with stepwise increasing

concentrations in styrene-methacrylate (Fritz 1989). Final embedding was in gelatin capsules in 100% plastic. All the steps of tissue processing were carried out under water-free conditions to prevent displacement or loss of diffusible elements in the root tissue. Polymerisation of the plastic-filled capsules took place in an oven with 60 °C over night and 45°C for 10 days.

After polymerization, semi-thin (<1 µm) sections of the root tips embedded in plastic capsules were cut using a ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria) with dry glass knives. The sections were mounted on hexagonal copper grids (Athene, provided by Plano, Wetzlar, Germany) with the adhesive decribed by Fritz (2007), coated with carbon and stored over silica gel.

5.2.4 Staining with Toluidin blue and light microcscopy

For observation of mycorrhizal structures semi-thin sections of root tips (0.5-1 mm thickness) were cut from the same blocks as used for X-ray microanalysis, stained with Toluidin blue, mounted with Euparal (Carl Roth KG, Karlsruhe, Germany) on glass slides and envisaged under the light microscope (Zeiss, Axioplan, Oberkochen, Germany). Sections were photographed with a digital camera (Zeiss AxioCam MrC, Software AxioVision Release 4.6.3).

5.2.5 X-ray microanalysis (TEM-EDX)

Semi-thin sections were analyzed by energy-dispersive X-ray microanalysis (EDX; EDAX DX-4, EDAX International Mahwah, NJ) under standardized conditions using a FEI Tecnai G² Spirit BioTWIN transmission electron microscope (TEM; FEI Company, Eindhoven, The Netherlands). This microscope is equipped with a Si (Li) detector with a thin beryllium window (8 mm thick).

For the measurements, in most cases a STEM magnification of 1200x was used. The dwell time for each measurement point was 10.000 ms in the live second mode. The take-off angle 15° tilt towards the detector. The accelerating voltage was 80 kV.

Subcellular element content in cross sections of plant roots and fungi was analyzed in beech and ash plant structures, and the fungal structures of beech (EcM) and ash (AM) roots. Ten replicates were analyzed in each of these compartments in three different root samples (n=30).

There were four different treatments (Table 1).

Tab. 1: Treatments of this experiment

species	EcM/AM	mix/mono
beech	EcM	mono
beech	EcM	mix
ash	AM	mono
ash	AM	mix

The spectra were analysed using the Tecnai Image Analysis (TIA)-Offline software (FEI Company, Eindhoven, The Netherlands). The spectra were automatically background fitted by the TIA-Offline software. Values of elements are given as background-fitted peak intensity. EDX spectra were collected between 0 and 10 keV. The peak centres of the different elements (K alpha) are Na 1.04 keV, Mg 1.25 keV, Al 1.48 keV, Si 1.74 keV, P 2.01 keV, S 2.31 keV, Cl 2.62 keV, K 3.31 keV, Ca 3.69 keV, Fe 6.38 keV and Cu 8.06 keV.
Data are means (+-SE). Statistical analysis of the data was performed using analysis of variance (ANOVA). Means were considered significantly different from each other, if the level of significance was $p \leq 0.05$.

5.3 Results and Discussion

5.3.1 Element concentrations in root tissues of beech and ash

Ash plant cells generally contained higher nutrient amounts than beech plant cells (Fig. 1 B). This is in line with the observation made by ICP analysis of roots in the Hainich area (Lang and Polle 2011). The two tree species differ in physiological traits like shade tolerance and root and canopy architecture. Ash has generally higher growth rate, a high foliar nutrient content and faster litter decomposability (<1 year) compared to beech, which is characterized by an intermediate growth rate, lower nutrient content in leaves and intermediate to slow leaf litter decomposition rates (3 years; Read, 1991; Cornelissen et al. 2001; Jacob et al. 2009). Because of these different plant host traits, it seems reasonable that ash trees have higher element contents in roots and subsequently a higher turnover of nutrients.
In beech root tissues, phosphorus (P) and potassium (K) were higher in the mixed plots with ash than in the monospecific plots (Fig. 1 A). Calcium (Ca) concentrations in beech root tissues were lower in mixed plots with beech and ash compared to beech mono plots. Element concentrations in ash root tissues were lower in the mixed plots with beech compared to ash mono plots (Fig. 1 B). This was the case for all five measured elements. Studies comparing monospecific vs. mixed tree stands showed that the rate of nutrient mineralization is higher in mixed stands compared to monospecific stands (Talkner et al. 2009; Richards et al. 2010).

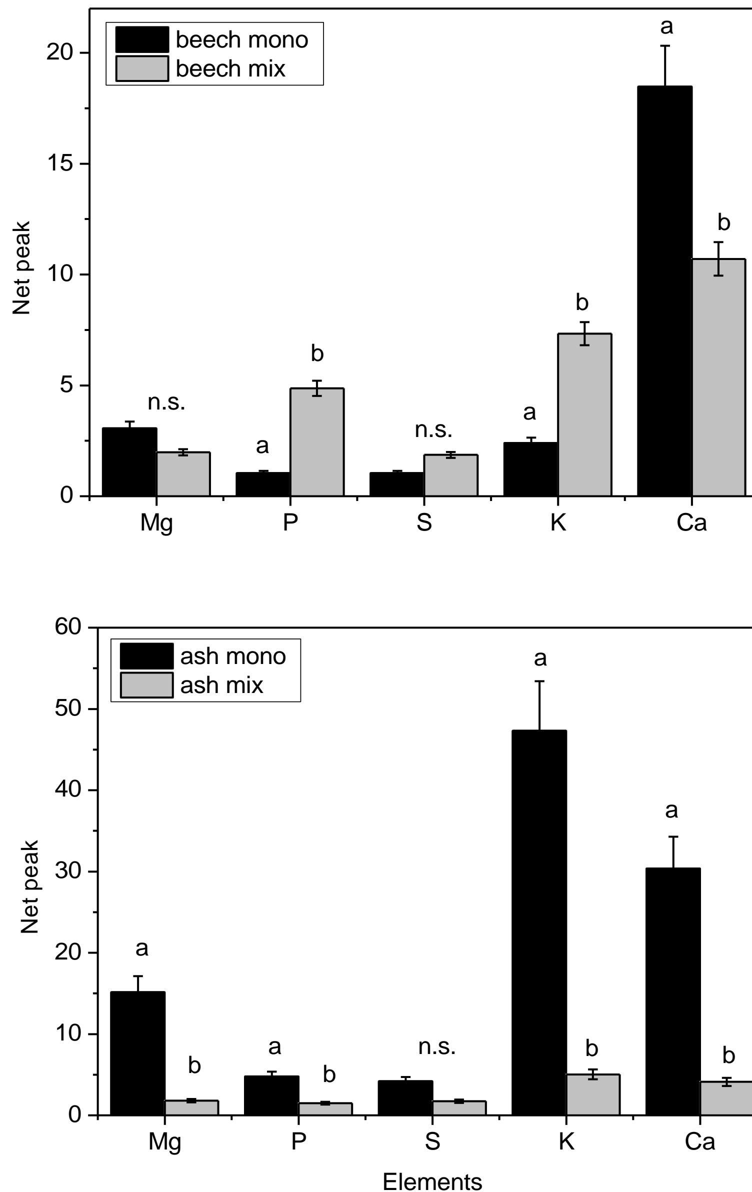


Fig. 1 A,B: Subcellular element concentrations in beech and ash roots. beech mono = pure beech plots. beech mix = mixed beech + ash plots, ash mono = pure ash plots, ash mix = mixed ash + beech plots. Mean values ± SE. Different letters indicate significant differences at p≤0.05. n.s. = not significant.

5.3.2 Element concentrations in fungal tissues of EcM and AM

The element levels in fungal tissues mirror in most cases the element levels in plant roots (Fig. 1 and 2). This is true for AM fungal tissues and ash plant roots, while nutrient amounts in EcM were slightly higher than in beech plant roots. EcM fungal tissues generally displayed lower element amounts compared to AM fungal tissues (2A,B).

In accordance with the results found in the root tissues of ash, the element concentrations of all five measured elements in AM fungal tissues were lower in mixed treatments compared to AM mono plots (Fig. 2 B). Element concentrations in EcM fungal tissues were not affected by neighbouring ash trees. We did not find a mono vs. mix treatment related significant difference for the measured element levels (Fig. 2 A) in EcM fungal structures. Only for calcium, slightly higher levels were found in EcM mono plots than in mixed plots (p= 0.0531). Mixture of beech and ash mainly affects nutrient concentrations in beech root tissues, but not in EcM fungal tissues.

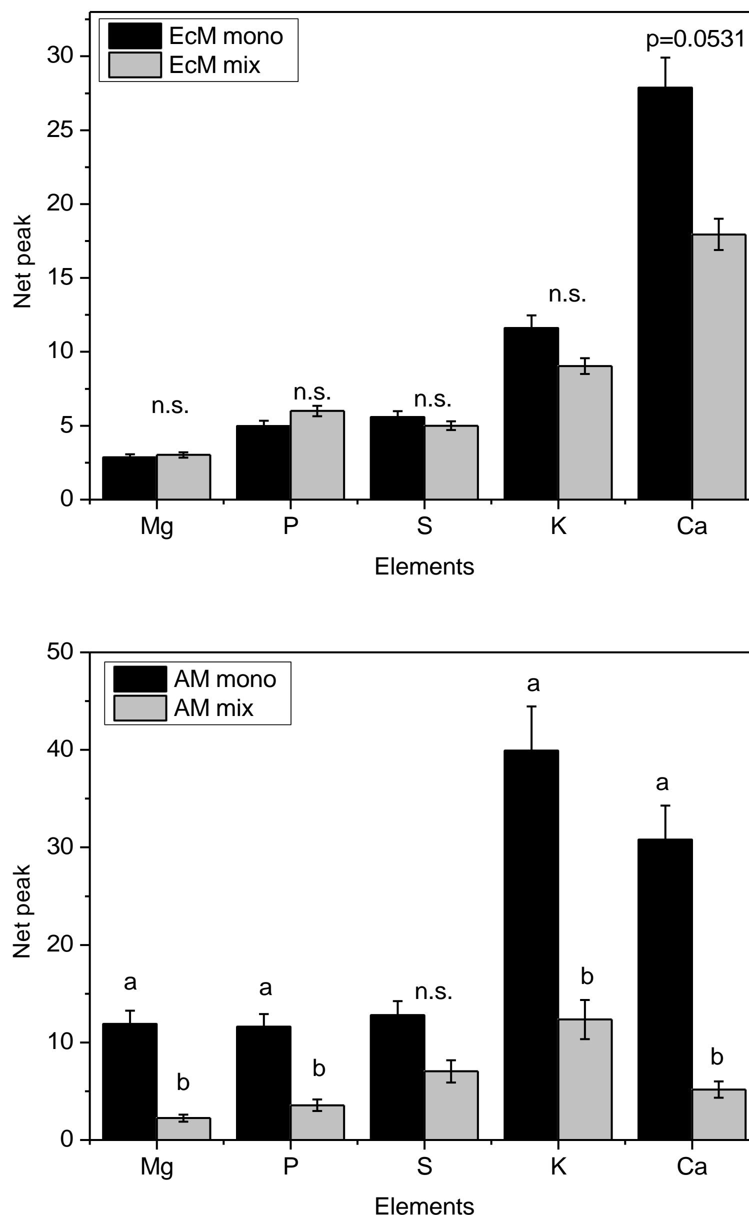


Fig. 2 A,B: Subcellular element concentrations in EcM and AM fungal tissues. EcM mono= pure beech plots. EcM mix = mixed beech + ash plots. AM mono= pure ash plots. AM mix = mixed ash + beech plots. Mean values ± SE. Different letters indicate significant differences at p≤0.05. n.s. = not significant.

The results of studies comparing mono vs. mixed stands may be influenced by the site conditions of monospecific vs. mixed stands. In our study, we analyzed the influence of neighbouring tree species on a mixed forest plot in the Hainich forest, where site conditions are well known (Guckland et al. 2009). In our study, we focused to individual tree species and the direct comparison of beech and ash trees, which includes several tree species related differences. Since these two tree species show completely different traits, effects are expected when these two tree species occur in mixture. Leaf litter of beech and ash trees differs regarding decomposition rate, pH and calcium levels (Jacob et al. 2009). Different nutrient composition of leaf litter of beech and ash trees influences soil nutrient status (Langenbruch et al. 2012), leading to a patchy distribution of soil nutrients (Hodge 2004).
Since resource availability is limited, it seems reasonable that interactions between tree individuals and between tree species occur. The presence of a tree neighbour most probably results in the competition for resources; i.e. water and nutrients. This competition can occur aboveground in the tree canopy as well as belowground, where roots interact and compete for space and resources and communicate via root exudates. Richards et al. (2010) reviewed studies which compared mixed vs. monospecific stands and reported that these studies showed a higher nutrient content in aboveground structures of species in mixed stands. It is argued that mixture of species leads to an increased uptake of resources from the mixed stand, suggesting higher rates of photosynthesis and greater resource-use efficiency (Richards et al. 2010). Such shifts can result from changes in canopy photosynthetic capacities, changes in carbon allocation or changes to foliar nutrient residence times of species in a mixture. Increased Ca concentrations in branches and stems and increased N, P, K, Ca and Mg in aboveground biomass in mixed stands compared to monocultures were found in a tropical plantation (Oelmann et al. 2010). However, information about influence of mixed stands on nutrient amounts in roots is scarce (Lang and Polle 2011). One of the few studies found higher element amounts in pure beech roots compared to young and older mixtures of beech and pine, but roots of pure pine stand had lower elements than the mixtures (except calcium; Rumberger 2004). The reasons remain unknown, and this study showed differences between root tissues of tree species and tissues of mycorrhizal types (EcM vs. AM).

5.4 Acknowledgements

This study was conducted in the context of the graduate school 1086 'The role of biodiversity for biogeochemical cycles and biotic interactions in temperate deciduous forests'. We thank the National Park administration for the permission to perform the study in the Hainich National Park and the German Research Council (DFG) for funding. We thank our colleagues for help with sample collection and Dr. Andrea Olbrich for technical advice during embedding of samples and TEM-EDX measurements.

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CHAPTER

6

Synopsis

6.1 Factors that shape mycorrhizal communities

In this study, it was found that leaf litter species (ash vs. beech leaf litter and mixtures of both species) influenced mycorrhizal community composition on root tips, while length of extractable hyphae was not influenced by the overlaying leaf litter layer (Chapter 2). EcM species differed in ^{15}N assimilation from labelled leaf litter (Chapter 3), which might indicate functional differences between mycorrhizal species regarding nutrient acquisition.

The leaf litter of ash and beech trees differs in chemical properties like decomposition rate, lignin content, nitrogen and nutrient content (Jacob et al. 2009). Ash leaf litter is completely decomposed after 1 vegetation period, while beech leaf litter has an average decomposition time of 3 years (Read, 1991). The diverse chemical environment created through application of heterogeneous leaf litter inputs influences mycorrhizal community and nutrient cycling in this study. The change of community composition after application of different leaf litter suggests functional diversity of mycorrhizal species because mycorrhizal species which decline after changed leaf litter may not be adapted to the changed soil conditions, while the relative abundance of mycorrhizal species which are better adapted to the new soil conditions increases.

Arbuscular mycorrhizal (AM) and ectomycorrhizal (EcM) fungi are adapted to different environments, with respect to plant type, humus type etc. (Read 1991). Different species of EcM and the AM-species *Glomus* on ash were shown to differ in subcellular element concentrations (Chapter 4), and this element concentrations in ash and beech root tissues and AM fungal structures was shown to be influenced by the presence of a heterospecific neighbouring trees species (Chapter 5).

Therefore, it seems reasonable that introduction of ash trees into a beech stand has implications for forest ecosystem processes, soil properties, rhizosphere organisms (e.g. Langenbruch et al. 2012; Cesarz et al. 2013) and subsequently also to mycorrhizas.

6.2 Functional traits of the mycorrhizal species of this study

The findings of this study support that mycorrhizal species differ in their functional roles regarding nutrient uptake, transfer and storage. It is therefore of interest to discuss the novel findings in the light of known traits of these organisms..

6.2.1 *Cenococcum geophilum*

Root tips colonized by the ascomycete *Cenococcum geophilum* are unramified and characterized by a warty black mantle with frequent emanating hyphae. *Cenococcum geophilum* belongs to the short distance (SD) exploration type (Agerer 2001). It is one of the most dominant and abundant mycorrhizal species in temperate and boreal forests (Horton and Bruns 2001). While the fungus does not form fruitbodies, a high abundance of sclerotia, i.e. resting structures of the fungus, in soil was reported (Dahlberg et al. 1997). *Cenococcum geophilum* shows a remarkably high resistence to decomposition (Fernandez et al. 2013), which was ascribed to its melanin content in the cell walls.
In several studies a high resistance to drought stress compared to other EcM fungal species has been reported (Coleman et al. 1989; Mexal and Reid 1973; Pigott 1982a; 1982b). Glucose respiration in relation to soil water potential was less altered in *Cenococcum* compared to *Lactarius* sp. (Jany et al. 2003). It was assumed that *Cenococcum geophilum* is a species complex (Dickie 2007). A high degree of genetic diversity for *Cenococcum geophilum* strains was reported (Jany et al. 2002). Seasonal variation of this species, being most abundant in the dry summer periods (Buée et al. 2005) support its role as a drought-tolerant fungus. The abundance of *Cenococcum geophilum* correlated with the soluble amino acid uptake (Dannenmann et al. 2009).
In our study *Cenococcum geophilum* showed a strong decline after application of ash leaf litter in a beech forest (Chapter 2). Since the species is abundant in the Hainich forest (9.58 % relative abundance; Lang et al. 2011), as well as after exposure to pure unlabelled beech leaf litter (21.5 % relative abundance), it appears to be adapted to low N. Application of ash leaf litter also changes humidity of soil and litter. Therefore an influence of humidity may also be responsible for the decline of *Cenococcum*.
In the few samples that were present, high ^{15}N values (16 ‰ δ ^{15}N) in the unlabelled treatments were measured, which is in line with the high ^{15}N observed by Lilleskov et al. (2002), but not with other studies (Haberer et al. 2007). Element analyses (Chapter 4) in this thesis showed a high sulphur content in *Cenococcum geophilum* cell walls of the hyphal mantle than in other EcM. Lang (2008) reported also a high sulphur content in beech roots when colonized by *Cenococcum geophilum*. The melanin content of the fungal cells walls, which protects the fungus from decomposition and leads to high persistence in soil (Fernandez et al. 2013). Melanin is a polymer which contains sulphur, therefore this could lead to the high sulphur content in the fungus.

6.2.2 *Lactarius subdulcis*

Lactarius subdulcis has a smooth orange mantle surface, is hydrophil and belongs to the contact exploration type with only few emanating hyphae (Agerer 2001). It is one of the most dominant mycorrhizal species in temperate forests. Here it showed a relatively constant abundance of 10-30 % across different leaf litter treatments (Chapter 2) and was obviously not affected by the application of ash leaf litter. *Lactarius* species are mostly tolerant to elevated N and also are variable protein users (Lilleskov et al. 2011). *Lactarius* has the ability to degrade complex phenolics (Rineau and Garbaye 2009). In our study *Lactarius subdulcis* showed high N concentrations (3 % N) compared to the other EcM investigated, and a stable relative contribution to ^{15}N accumulation of 22 % in May as well as in October. Root tips colonized by *Lactarius* had intermediate natural abundance ^{15}N values compared to *Cenococcum* and *Xerocomus*, and similar values compared to *Russula* (Haberer et al. 2007), which is in line with ^{15}N assimilation reported in this study (Chapter 3).

6.2.3 *Russula* sp.

Russula species are dominant EcM fungi in many forest ecosystems (Horton and Bruns 2001; Peter et al. 2001) with a high diversity and strong habitat partitioning (Geml et al. 2010). *Russula ochroleuca*, one species of this study, is characterized by a white surface with wooly hyphae and unramified colonized root tips. *Russula ochroleuca* belongs to the contact exploration type and is hydrophilic (Courty et al. 2008). *Russula* was classified as a “protein-fungus”because of its abilities to use organic N from protein (Lilleskov et al. 2002; 2011). It has been reported that the abundance of *Russula* species decreases when N increases (Wallenda and Kottke 1998; Peter et al. 2001). Other studies showed that *Russula* produced five times more sporocarps in an N deposition treatment than at low N availability (Avis et al. 2003). In this study relative abundance of *Russula ochroleuca* increased from May to October, especially in the mixed leaf litter treatments (Chapter 2). In this study, ^{15}N values of mycorrhizal root tips colonized by *Russula ochroleuca* showed the highest ^{15}N assimilation in October compared to the other EcM species. Other studies found that *Russula* was intermediate in ^{15}N natural abundance compared to *Cenococcum* and *Xerocomu*s (Haberer et al. 2007). Pena et al. (2013) found *Russula cuprea* to accumulate the lowest ^{15}N compared to other EcM fungal species.

6.2.4 *Xerocomus* sp. (*Xerocomus pruinatus* / *Xerocomus sp*.)

All *Xerocomus* species belong to long distance exploration type (Agerer 2001) and have hydrophobic rhizomorphs. *Xerocomus badius* shows high activity and element storage capacity (Kottke et al. 1998) and best growth on nitrate than other EcM (Nygren et al. 2008).

Xerocomus pruinatus showed an ability to mobilize N from protein and to degrade complex phenolics (Rineau and Garbaye 2009). Response to elevated N is mixed, ranging from positive, no change to negative responses (Lilleskov et al. 2011). In our study *Xerocomus* increased massively in relative abundance after ash leaf litter application, from a long-term average of 5 % relative abundance (Lang et al. 2011) to 30 % in mixed ash-beech leaf litter treatments (Chapter 2). High ^{15}N accumulation was found after exposure to N rich labelled ash leaf litter (Chapter 3). *Xerocomus* species belong to the *Boletaceae*, which have proteolytic abilities and exhibit high ^{15}N natural abundance signature (Lilleskov et al. 2002; Taylor et al. 2003; Hobbie and Agerer 2010). *Xerocomus* did not have primary access to N from labelled beech leaf litter, but a significant ^{15}N accumulation with long delay was reported (Pena et al. 2013). Low ^{15}N natural abundance compared to *Cenococcum*, *Lactarius* and *Russula* was shown (Haberer et al. 2007), probably because of the delay in ^{15}N accumulation reported by Pena et al. (2013).

6.2.5 *Clavulina* sp. (*Clavulina cristata*)

Clavulina cristata belongs to the medium distance exploration type (Courty et al. 2008) and is characterized by a hydrophilic, white to grey-brown, sometimes velvet mantle surface (Buée et al. 2005). It occurs in deciduous and coniferous forests (Peter et al. 2001) and was reported to exhibit seasonal variation, being most abundant in the winter and spring (Buée et al. 2005; Courty et al. 2008). The mycorrhizal life style of this fungus is now confirmed (Buée et al. 2005; Tedersoo et al. 2010). It was classified to be tolerant to low temperature and drought resistant (Buée et al. 2005). Peter et al (2001) found no effect of N addition on *Clavulina cristata*, and concluded that this fungus is insensitive to N. Sporocarp ^{15}N has been reported to be around 2 δ ‰ ^{15}N (Hobbie et al. 2001). To our knowledge, no data about ^{15}N on root tips colonized by *Clavulina cristata* are available, our data show a mean value of 3.74 δ ‰ ^{15}N in unlabelled samples in May and the highest relative contribution to ^{15}N assimilation in May compared to the other EcM species of this study (Chapter 3). In October, relative abundance of *Clavulina* declined (Chapter 2).

6.2.6 *Tomentella* sp. (*Tomentella castanea* / *Tomentella botryoides*)

Tomentella species belong either to the short distance or contact exploration type (Agerer 2001). *Tomentella* EcM are brown, unramified and have a smooth surface with few emanating hyphae (Buée et al. 2005) and are dominant EcM in temperate forests (Horton and Bruns 2001). *Tomentella sublilacina* was classified as a late-successional fungus (Lilleskov and Bruns 2003). The authors concluded that this fungus colonizes the root slowly and has a greater competitive ability than other EcM fungi. In our study, leaf litter composition did not lead to a strong change in relative abundance of *Tomentella castanea* and *Tomentella botryoides*, but their relative abundance was higher in spring than in fall (Chapter 2). While our study found rather low ^{15}N accumulation compared to other EcM fungal species (Chapter 3), *Tomentella badia* exhibited the highest degree of ^{15}N enrichment from leaf litter and a strong reaction to removal of leaf litter bags (Pena et al. 2013). *Tomentella* showed high ability to produce enzymes involved in litter decomposition (Köljalg et al. 2000).

6.2.7 *Thelephora* spec. (*Thelephora terrestris*)

Thelephora terrestris belongs to medium distance exploration type (Courty et al. 2008) with very few emanating hyphae and a white to brown smooth surface. *Thelephora terrestris* was reported to have saprotrophic abilities with crusts of log or leaf litter (Peter et al. 2001). It is able to use protein (Lilleskov et al. 2011). Increasing N deposition led to an increase of *Thelephora* (Peter et al. 2001; Cox et al. 2010) or had no effect (Wallenda and Kottke 1998). In this study *Thelephora* showed high relative abundance in the unlabelled beech treatment in spring, and in the pure leaf litter treatments in fall, but massively declined in mixed leaf litter treatments in October (Chapter 2). The species showed the lowest N concentration (around 1 % N) but relatively high ^{15}N accumulation compared to the other EcM of this study (Chapter3).

6.2.8 *Amanita rubescens*

Amanita rubescens is characterized as a medium distance exploration type, with irregularly ramification and a brown-red colour. It was shown to decrease (Peter et al. 2001) or increase (Cox et al. 2010) after exposure to elevated N. In our leaf litter experiment the fungus showed a low relative abundance, was nearly not present at all in spring, but increased in fall in treatments with ash leaf litter (Chapter 2). The relatively low ^{15}N of 1 ‰ found in the study of Hobbie et al. (2001) corresponds with the relatively low ^{15}N assimilation in our study (Chapter 3). Hobbie and Högberg (2012) reviewed that *Amanita* species have similar ^{15}N natural abundance values as *Lactarius* and *Russula* species.

6.2.9 *Glomus* sp.

In our study *Glomus sp.* showed higher element concentrations compared to the EcM species (Chapter 4). This is contradictory to studies where EcM were shown to be more effective for P uptake than AM, when colonizing the same host plant (Jones et al. 1998; Egerton-Warburton and Allen 2001). The high element concentrations of *Glomus sp.* were affected when a neighbouring beech trees occured (Chapter 5). Studies on the functional differences between AM fungal species are much more rare than those on EcM fungi (Feddermann et al. 2010). However, AM fungi (*Glomus*, *Acaulospora*, *Scutellospora*) differ in the ability to take up and transfer P to cucumber seedlings (Jakobsen et al. 1992 a;b). *Acaulospora laevis* shows a greater ability for P transfer than *Glomus* sp. or *Scutellospora calospora*, which might also been related to differences in spread of the ERM of the species. AM were shown to vary in their strategy to obtain soil P (Jansa et al. 2005); and when *Glomus claroideum* and *Glomus intraradices* colonized the same root system of leek plants, plants acquired more P than with one of the two species alone (Jansa et al. 2008). It was shown that *Scutellospora calospora* used clearly more host carbon and had a significantly lower P:C exchange ratio than *Glomus caldonium* on cucumber plants (Pearson and Jakobsen 1993). This was attributed to the fact that formation of spores in the *Glomaceae* is very quick (4 weeks) compared to *Gigasporaceae*, since the spores of *Gigasporaceae* are much larger. Length and density of ERM and colonized root length of *Glomus mosseae* and *Glomus intraradices* were analyzed and were positively correlated with growth, phosphorus (P) and nitrogen (N) nutrition of *Medicago sativa* plants grown in microcosms (Avio et al. 2006). Two *Glomus* species showed high and *Gigaspora rosea* only low hyphal growth; and *Glomus intraradices* was more successful in delivering P to three plant species while *Glomus caledonium* and *G. rosea* delivered less P. The amount of delivered P was not related to colonisation or to growth or P responses (Smith et al. 2004). There is high variation between AM species in root colonization, and there is high variation in growth responses between several host plants and AM fungal species (Smith et al. 2011).

6.3 Taxanomic vs. functional diversity – does diversity matter?

The data presented here support that several EcM species as well as one AM species (*Glomus* sp.) differ in their functional roles for nutrient assimilation and storage. One host plant is colonized by a variety of mycorrhizal species, the sum of total benefits is not easy to determine. The high biodiversity of mycorrhizal fungi is thought to be linked to a broad range of of functional abilities, which in turn is important for ecosystem functioning (Cairney 1999; Leake 2001; Koide et al. 2013), since a reduction of fungal diversity might in turn have consequences for ecosystem functioning. The response to environmental conditions varies quickly in natural forest ecosystems, where complexity of soil influences rhizosphere dynamics and interactions between several organisms (Buée et al. 2009). It would be interesting to be able to predict how the mycorrhizal community is composed in a given environment, and to predict how a change in environmental variables influences this community composition and abundance of certain species (Koide et al. 2009). More research in the field is necessary to determine the variable functions of mycorrhizal fungi on the long term, especially for arbuscular mycorrhizal fungal species.

6.4 References

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Declaration of originality and certificate of authorship

I, Jasmin Seven, hereby declare that I am the sole author of this dissertation entitled "Mycorrhizal fungi in deciduous forests of differing tree species diversity and their role for nutrient transfer". All references and data sources that were used in the dissertation have been appropriately acknowledged. I furthermore declare that this work has not been submitted elsewhere in any form as part of another dissertation procedure. I certify that the manuscripts presented in chapters 2, 3 4 and 5 have been written by me as first author.

Göttingen, Juni 2014

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Acknowledgements

The work for this thesis was performed at the Department of Forest Botany and Plant Physiology of the Georg-August University of Göttingen. Without the manifold support of many people, finishing this PhD thesis would have been impossible.

First of all, I would like to thank my main supervisor Prof. Dr. Polle for giving me the opportunity to work on this very interesting and sometimes challenging topic, for the constant support and encouragement, fruitful discussions and for the possibility to expand my horizon on several conferences and courses all across Germany and Europe. She constantly supported me with dose of freedom, what I appreciate.

Furthermore, I thank the members of my thesis committee for their time and effort, especially Prof. Dr. Leuschner for reviewing my PhD thesis.

I thank the DFG for funding of my project in the frame of the Graduiertenkolleg 1086. Furthermore I would like to thank the National Park Service for the unique opportunity to research in the Hainich National park.

Special thanks go to Dr. Christa Lang, who introduced me into the mycorrhizae of the Hainich forest and to Dr. Andrea Olbrich, who introduced me into the EDX-technique, transmission electron microscopy and the mysteries of embedding in plastic. Your door was always open whenever I needed advice and I thank you both so much for your countless help and support and for being so friendly, warm and patient with me.

Thank you very much to Dr. Rosemarie Langefeld-Heyser, who raised my enthusiasm and passion about science, and showed me that botany and plant anatomy is not boring.

Special thanks go to Dr. Kerttu Valtanen, my office mate, for many funny finnish moments and lots of laugh, and for being always there and becoming a very good friend.

Our small mycorrhiza-PhD Group, especially Christa, Kerttu, Dr. Lara Danielsen and Michaela Rath, I thank for lots of discussions, help, root washing and morphotyping times together. Lara made the fungi conference in Edinburgh a great experience.

Thank you very much to Dr. Christina Langenbruch, who provided leaf litter data, and to Dr. Verena Eissfeller and Andrea Scheibe for the excellent teamwork during our labelling experiment. Even in the snow nothing could hold us back and I always had a great time with you.

I am especially thankful to all PhD students of the ‘Graduiertenkolleg 1086’ for having a great time in the Hainich and in Göttingen and for being great company, especially Christina, Dr. Ellie Vockenhuber, Dr. Nicole Legner, Dr. Andreas Jacob and Dr. Kristin Schröder.

To all my colleagues of the Departement of Forest Botany and Plant Physiology in Göttingen (FBI) I am deeply grateful for the friendly and supportive working atmosphere and the constant support over the last 10 years. Especially I would like to thank Gisbert Langer-Kettner, Christine Kettner, Merle Fastenrath, Sabine Wolfarth, Dr. Thomas Teichmann, Dr. Urs Fischer, Marianne Smiatacz,

Viktoria Pfander, Karin Lange, Gaby Lehmann and Monika Franke-Klein for help and support in the lab and in the greenhouse, Thomas Klein for help with molecular work and Bernd Kopka for help with my computer problems. Several colleagues helped during sampling dates, especially Dr. Peter Hawighorst, Dr. Dennis Janz, Rainer Schulz, Nils Hoffmann and Konstantin Knorr. I really enjoyed working with all colleagues and PhD students and I thank you for nice coffee and lunch breaks and evenings in Sausalitos.

The staff of the KOSI is gratefully acknowledged for measuring my vast number of samples quickly and reliably and for answering all my stupid questions.

Meinen lieben Freunden aus Braunschweig und Göttingen danke ich für die Unterstützung und das Verständnis für mich die letzten Jahre, wenn Besuche und Anrufe immer seltener wurden. Judith Wassiltschenko danke ich dafür, dass sie die Buchen-Eschen-Kombinationen mit mir gefunden hat.

Meinem Ehemann Hauke danke ich für die Unterstützung und die Geduld mit mir die ganzen Jahre. Ohne deine vorzüglichen Kochkünste wäre ich in den letzten Monaten sicherlich verhungert. Thomas Egill danke ich dafür, dass jeder Tag mit einem Lächeln beginnt. Kiste danke ich für die 8 Kilo in 16 Jahren. Meiner Familie danke ich einfach dafür, dass sie alle da sind; für die Unterstützung, die vielen aufmunternden Worte, Telefonate und Treffen in fröhlicher Runde. Besonders meinen Eltern, meiner Schwester Jessi und Carsten, Familie Jürgens, Oma Silvia, Opa Dieter und Oma Putzi danke ich für die Unterstützung, ohne die mein Studium sicher nicht möglich gewesen wäre.

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