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# Functional diversity of mycorrhizal fungi with regard to nutrient transfer

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## Summary

Mycorrhizal associations of tree species are important drivers of plant belowground interactions. The main objective of this study was to investigate the contribution of mycorrhizal fungi on plant competition for nutrients. Another goal of the present study was to determine nutrient and carbon fluxes between trees, and soil food web via mycorrhizal fungi.

The influence of interspecific interactions on N and P acquisition of ash (*Fraxinus excelsior*) and beech was analysed in nutrient limited conditions. Beech and ash saplings were grown in conspecific and heterospecific pairs and labelled with nutrient solution containing 6.27 ng $^{33}PH_3{}^{33}PO_4$ (3.7 MBq) and 4 mM $^{15}NH_4{}^{15}NO_3$. The growth of beech was not influenced by the species identity of the neighbouring tree, whereas the height growth of ash decreased in the presence of beech. Beech was also neutral to interspecific competition for nutrients, whereas ash shifted to increased deprivation of N and P in the presence of beech.

The N and P accumulation was higher in EM root tips than in vital ash roots and non-mycorrhizal beech roots. Non-mycorrhizal beech root tips accumulated 1.2 times less N and 4.2 times less P than mycorrhizal root tips. Vital ash fine root tips accumulated 2.3 times less N and 6.7 times less P than mycorrhizal beech root tips. The N and P concentrations of beech fine roots and mycorrhizal root tips were positively correlated.

Differences in N and P accumulation of EM species demonstrated the functional diversity within beech roots colonizing EM community. The most abundant EM species *Tomentella castanea* and *Sebacina* sp. (81.7 % mono; 89.2% mix) had high P and N accumulation.

The ability of tree species to use organic N and carbon (C) was examined using $^{15}N$ and $^{13}C$ enriched litter in double-split-root rhizotrons planted with ash and beech saplings (Chapter 4). Nitrogen uptake from litter was documented in both ash and beech. No C from organic origin was detected in fine roots or other plant tissues after 475 days of incubation. Although beech root biomass was significantly lower than that of ash only beech decreased soil carbon and nitrogen concentrations significantly. These results suggest that trees, which are colonized by mycorrhizal fungi, use organic nutrient sources. However, the allocation of C is presumably unidirectional, namely from plant to fungus.

In another experiment we demonstrated that when the allocation carbon from photoassimilates was inhibited through girdling, EM was supplied from root carbon storages (Appendix, Chapter 6).

The path of plant derived C via EM fungi to soil food web was analyzed using $^{15}NO_3{}^{15}NH_4$ and $^{13}CO_2$ labelling. Beech saplings from Hainich national park were removed with intact soil cores in order to maintain intact soil community and labelled for five months in a green house. The highest concentrations of $^{13}C$ and $^{15}N$ were found in mycorrhizal root tips. The strong relation of $^{15}N$ in EM root tips and adjacent fine root demonstrated that $^{15}N$ taken up by the EM fungus was mainly transported to host plant. The results demonstrated that mycorrhizal structures are an important nutrient source for soil animals and a considerable channel of plant C into soil food web.

Based on the present results, the interactions between beech and ash are suggested to be driven mainly by effects of beech and associated EM fungi. It can be concluded that abundant root colonizing EM fungi significantly contribute to N and P nutrition of beech.

## II List of abbreviations

| | |
|---|---|
| µ | Micro ($10^{-6}$) |
| °C | Degree Celsius |
| *A* | Activity |
| AD | Average diameter |
| AM | Arbuscular mycorrhiza |
| ANOVA | Analysis of variance |
| As | Ash (*Fraxinus excelsior* L.) |
| Be | Beech (*Fagus sylvatica* L.) |
| Bq | Bequerel |
| c | Centi ($10^{-2}$) |
| d | Day |
| df | Degrees of freedom |
| dw | Dry weight |
| *E. coli* | *Escherichia coli* |
| EM | Ectomycorrhiza |
| *et al*. | *et alii* |
| FoA | Forestry office |
| FR | Fine root |
| g | Gramm |
| h | Hour |
| ha | Hectar |
| ITS | Internal transcribed spacer |
| k | Kilo ($10^{-3}$) |
| l | Litre |
| m | Meter |
| m (prefix) | Milli ($10^{-3}$) |
| M | Molar |
| M | Mega ($10^{6}$) |
| min | Minute |
| mix | Mixture |
| MN | Mycorrhizal network |
| mono | Monoculture |
| *n* | Amount of substances |
| n | Nano ($10^{-9}$) |
| NCBI | National Center of Biotechnology Information |
| Nds. | Niedersachsen |
| NM | Non-mycorrhizal |
| PAR | Photosynthetically active radiation |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| ppm | Parts per million ($10^{-6}$) |
| RAG | Relative annual height growth |
| RCI | Relative competition intensity |
| s | Second |
| SA | Surface area |
| SE | Standard error |
| SLA | Specific leaf area |
| SRL | Specific root length |
| SSA | Specific surface area |
| *t* | Time |
| *v* | Uptake rate |
| *vs.* | *versus* |

# Chapter 1

# GENERAL INTRODUCTION

# 1. Mycorrhizal symbiosis

## 1.1 Types of mycorrhizal associations

Mycorrhiza (*mýkēs* ,fungus, ῥίζα *rhiza*, root) is a symbiosis between specialised soil fungi and higher plants. Approximately 90% of terrestrial plants form mycorrhizal associations (Trappe 1987).

On the basis of morphological characteristics, mycorrhizal types can be divided into seven main groups: arbuscular mycorrhiza, arbutoid mycorrhiza, ectendomycorrhiza, ecto-mycorrhiza, ericoid mycorrhiza, monotropoid mycorrhiza and orchid mycorrhiza (Harley & Smith 1983). Tree species in European temperate forests form different types of mycorrhizal associations, whereof EM is the most prevalent (Tab.1).

**Table 1:** Examples of mycorrhizal types of common tree species in temperate forest. EM ectomycorrhizal, AM arbuscular mycorrhizal (Cornelissen et al. 2001, Dučić et al. 2009, Lang et al. 2011, Schüßler 2009).

| Deciduous tree species | | | Coniferous tree species | | |
|---|---|---|---|---|---|
| Species | Family | Mycorrhizal type | Species | Family | Mycorrhizal type |
| *Fraxinus excelsior* | Oleaceae | AM | *Pseudotsuga* spp. | Pinaceae | EM/Ectendo |
| *Acer* spp. | Aceraceae | AM | *Picea abies* | Pinaceae | EM |
| *Fagus sylvatica* | Fagaceae | EM | *Abies alba* | Pinaceae | EM |
| *Quercus* spp. | Fagaceae | EM | *Larix decidua* | Pinaceae | EM |
| *Salix spp.* | Salicaceae | AM/EM | *Pinus sylvestris* | Pinaceae | EM |
| *Populus* spp. | Salicaceae | AM/ EM | *Taxus baccata* | Taxaceae | AM |

The mycorrhizal types contribute differentially to functional traits of plant carbon (C) cycling (Cornelissen et al. 2001). In temperate forests, plants associated with AM display comparatively high growth rates, high foliar nutrient content and fast litter decomposition, whereas trees with EM have intermediate growth ratio, lower foliar nutrient contents and intermediate to slow litter decomposability (Cornelissen et al. 2001).

1.2 *Arbuscular mycorrhiza*

On basis of 400 million years old fossil funds arbuscular symbiosis (AM) is the oldest type of mycorrhiza. AM are formed by ca. 180 fungal taxa of the genus Glomeromycota (Smith and Read, 2008). In AM associations, fungal hyphae penetrate cortical cells of plant fine roots and develop hyphal coils and arbuscles to increase the surface area for the exchange of nutrient minerals and water with carbon. Several fungal species also form vesicles, structures used as lipid storages (van Aarle & Olsson 2003).

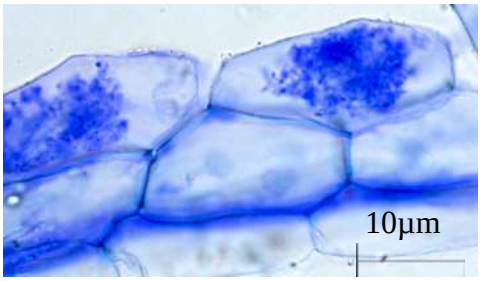


**Figure 1:** AM arbuscles

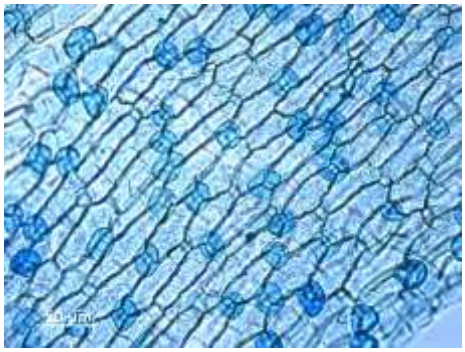

**Figure 2:** AM vesicles and hyphae

1.3. *Ectomycorrhiza*

The dominating mycorrhizal type in boreal and temperate forests is ectomycorrhiza (EM). Fossile records of EM originate from 50-52 million years ago (Beimforde et al. 2011, LePage et al. 1997), but presumably EM evolved together with gymnosperms and exist since 190 million years (Taylor et al. 2009). EM associations are formed by estimated 7000-10 000 fungal taxa and by 8000 plant taxa (Taylor & Alexander 2005). Even though only a small fraction of terrestrial plants form EM symbioses, they include numerous ecologically and economically important tree species (Taylor and Alexander, 2005).


**Figure 3:** EM mycorrhiza

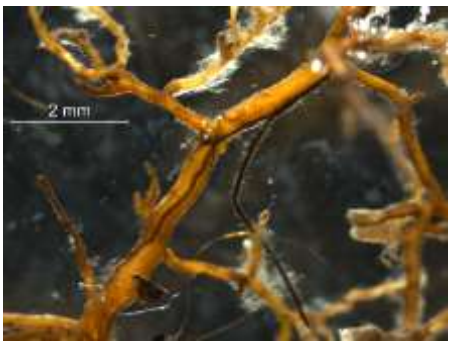


**Figure 4:** EM rhizomorphs on beech fine roots

EMs have an often well developed periradical phase, present as a hyphal mantle that covers the outside of the fine root tips and has a characteristic appearance, so called morphotype. The intraradical part of EMs consists of the Hartig net, an interface for plant carbon and nutrient exchange (Corrêa et al. 2012). The extraradical hyphae emanate from the mantle into the surrounding soil. They can greatly differ in structure, abundance and length and in some EM form vessel like structures, so called rhizomorphs, which serve the long distance nutrient and water transport (Agerer 1990).

### 1.4 *Functions of mycorrhizal association*

Mycorrhizal fungi provide their host plant with nutrients and water and in exchange up to 22% of the plant assimilated C (Hobbie 2006). When the allocation of recent photpassimilates to roots is restricted, trees have been shown to supply mycorrhiza from root C storages (Appendix 2). Recent experiments with $^{13}CO_2$, $^{15}N$ and $^{32}P$ labelling (Fellbaum et al. 2012, Kiers et al. 2011) and a meta analysis of published data (Corrêa et al. 2012) showed that in AM and EM associations, both plant and fungus control the nutrient and C exchange. When colonized with multiple fungi, plants provide beneficial mycorrhizal partners with more C than unprofitable partners. In turn, fungi can drive the symbiosis by increased nutrient transfer to roots containing higher C concentrations. The bi-directional control presumably results in a fair reciprocal transfer of nutrients and carbon (Corrêa et al. 2012, Kiers et al. 2011).

## 2. Nitrogen and phosphorus in plant nutrition

The most plant growth limiting factor in temperate forests is nitrogen (N) (Rennenberg et al. 1998). It is an essential component of numerous organic compounds of plant cells, such as amino acids, proteins and nucleid acids. Plants acquire N mainly from two sources: from soil, through mineralization of organic matter, as well as through natural and anthropogenic N deposition, and from atmosphere through symbiotic $N_2$ fixation (Vance 2001). In forest soils, the main fraction of N occurs in organic compounds, such as leaf litter, dead roots and soil organisms. Only a small fraction of soil N is present as ammonium ($NH_4^+$), nitrate ($NO_3^-$) or in form of simple amino acids that can be directly taken up by plant roots (Näsholm et al. 2009). Tree species differ in their preferences to distinct N forms (Schulz et al. 2011). Whereas coniferous tree species are generally considered to prefer $NO_3^-$ over $NH_4^+$, in deciduous species considerable species specific differences and contradictory results within one species have been reported (Dannenmann et al. 2009). In general, the uptake of $NH_4^+$ is considered as

more cost efficient, whereas $NO_3^-$ might be more plant available due to its higher diffusion rate and low absorption to soil particles (Darrah et al. 1983).

The second most plant growth limiting macronutrient in temperate forests is phosphorous (P) (Cairney 2011). For plants it is essential as a structural component of proteins, enzymes and nucleid acids, with numerous functions in plant metabolism and growth, such as photo-synthesis, respiration, as well as energy production, storage and transfer. In soils, both inorganic and organic forms of P exist. Organic P ($P_o$) is mainly present as phosphate monoesters, phosphate diesters and inositol phosphates, whereas inorganic P ($P_i$) is mainly present in form of mineral and dissolved phosphates (Schachtman et al. 1998). Although bound P is relatively abundant in many soils, the main portion of P is not available for plant uptake due to the high reactivity with other chemical and biological compounds (White and Hammond 2005). P availability varies with factors such as soil age, rates of mineral $P_i$ weathering, precipitation reactions with cations, rates of decomposition and immobilization in microbes and plants (Lambers et al. 2008). In Central European forests the amount of organic bound P increases with increasing tree species diversity whereas the ratio of mineral P is relatively constant irrespective of the tree species diversity (Talkner et al. 2009).

Plants take up mainly inorganic orthophosphate P*i*, which occurs in low concentrations (10µM) in the soil solution (Schachtman et al. 1998). The optimal P*i* uptake of most plants is recorded at soil pH between 6.0 and 5.0. Under these conditions P*i* mainly occurs as $H_2PO_4^-$. Due to the low diffusion rate ($10^{-12}$ to $10^{-15}$ $m^2$ $s^{-1}$) of P*i* in soil solutions, the phosphorous concentration in root near rhizosphere is rapidly depleted. In order to maintain plant internal P balance, plants store and recycle P (Schachtman et al. 1998).

Plants have evolved different strategies to overcome the nutrient depletion in the rhizosphere. These include the modification of root growth and architecture (Curt et al. 2005, Jacob et al. 2012), influence on soil chemical properties, which include acidification through proton extrusion and the release of root exudates (organic acids), as well as influencing soil microbial activity (Fender et al. 2012, Richardson et al. 2009). However, the most important strategy to overcome nutrient transport limitation in the depletion zone is the mycorrhizal association (Smith et al. 2001).

## 3. Functions of AM and EM in plant P and N acquisition

Numerous mycorrhizal fungi have been shown to substantially enhance both plant N and P uptake (Cairney 2011, Plassard & Dell 2010, Smith & Read 2008). The host plant receive

nutrients via mobilisation and absorption by fungal mycelia, translocation through fungal hyphae to the fungus-root interface and transfer across the fungus-root interface (Nehls et al. 2007). Both AM and EM fungi produce extraradical mycelia, that formed by AM can reach soil areas of several cm (Drew et al. 2003, Eissenstat 1990) and that by EM up to several meters (Fiore-Donno & Martin 2001) from root surface. Thus, both fungal types extend far beyond the nutrient depletion zone of the rhizosphere and generate an efficient network of nutrient uptake. Mycorrhizal root tips and hyphae produce a range of exudates that serve the nutrient release by mineral weathering (Landeweert et al. 2001), mineralisation of organic polymers (Durall et al. 1994, Read & Perez-Moreno 2003), and that are also required for nutrient uptake processes, metal detoxification and antimicrobial defence. Exudates consist mainly of low molecular weight organic acids, saccarides, amino acids and peptides but EM root tips also release fatty acids, polymeric carbohydrates and different enzymes into the soil (Courty et al. 2005, Gadd 2007).

In temperate forests, EM fungi contribute up to 80% of the host plant N (van der Heijden et al. 2008). EMs have been shown to be able to use both mineral N sources nitrate ($NO_3$) (Nygren et al. 2008) and ammonium ($NH_4$), most likely with a preference to ammonium, if both N forms are available (Finlay et al. 1989, Smith and Read 2008). Furthermore, EMs have been reported to use a range of organic compounds, such as proteins and nucleic acids, as N sources (Marmeisse et al. 2004). They secrete extracellular proteinases and peptidases that effectively hydrolyse organic N sources to amino acids, which can be absorbed by the fungus (Chalot & Brun 1998, Nygren et al. 2008). The production of extracellular phosphor-monoesterases and phosphodiesterases has been reported, as well as that of hydrolytic enzymes such as cellulases and hemicellulases (Nygren & Rosling 2009). These enzymes might serve the penetration to dead organic material in soil for uptake of sequestered mineral nutrients (Morel et al. 2006, Nygren & Rosling 2009).

AM fungi have been shown to take up and transport $NO_3^-$ (Govindarajulu et al. 2005, Tobar et al. 1994) and $NH_4^+$ (Jin et al. 2005, Peréz-Tienda et al. 2012). The uptake of N from organic sources has been reported (Hawkins et al. 2000, Hodge & Fitter 2010), however it is not clear in which form AM acquire N from organic compounds (Hodge 2001, Leigh et al. 2009). AM fungi mainly take up mineral P, but some species have been shown to extract P from organic sources with extracellular phosphatases (Hodge & Fitter 2010, Jayachandran et al. 1992). Also EM are able to use both mineral and organic P sources (Cairney 2011). The absorption

of $P_i$ is maximized by high affinity transporters of the types $P_i$:$H^+$ and $P_i$:$Na^+$ (Harrison et al. 2002, Plassard & Dell 2010). Several AM and EM putative $P_i$ transporter genes have been reported, that are mainly expressed in extraradical mycelium during the symbiosis (Harrison & van Buuren 1995, Martin et al. 2008, Tatry et al. 2009).

In mycorrhizal hyphae, N is transported mainly as amino acids and $NH_4^+$, P presumably as $P_i$ (Chalot et al. 2006, Müller et al. 2007). P absorption is regulated by phosphate demand of the host plant. Plant P demand is reflected by plant P status, as well as by the concentration of hyphal polyphosphates, which serve as $P_i$ storage pools (Bücking et al. 1999, Bücking et al. 2000, Cairney & Smith 1992, Finlay 1989). At the fungus-root interface, the nutrients are transferred through efflux across the fungal plasma membrane (Bücking et al. 1999). Subsequently, nutrients are absorbed from the apoplasm of the fungus-root interface across the plasma membrane of the host root cell (Nehls et al. 2007). The apoplasmic compartment serves the control of local chemical and physical properties of the plant-fungus interface. This presumably results in an equal control of the exchange by both fungus and host plant (Corrêa et al. 2012, Kiers et al. 2011, Nehls et al. 2007).

## 4. Plant interactions

Plant competition occurs when individuals of same species (intraspecific) or different species (interspecific) compete for the same resource, such as light, nutrients or space. Interactions among plant individuals are usually size-asymmetric, which means a resource pre-emption by a larger individual, usually measured by disproportional size advantage of larger individuals of a population. In the aboveground compartment, this is comprehensible, since the main limiting resource is light. A taller plant can pre-empt light from shorter neighbours. Therefore, already a small size difference can offer a considerable competitive advantage. Similarly, investigations of fine root biomass distributions and competitive interactions in the field, which were investigated by soil core analysis and root growth chambers with isolated but intact fine root endings of mature trees, a markedly asymmetric belowground competition has been demonstrated in temperate forests (Leuschner et al. 2001, Rewald & Leuschner 2009). Since mycorrhizal interactions tightly associate with plant interactions (Jacob et al. 2012), it is important to consider them as a part of the plant interactions.

## 5. Mycorrhizal fungi in ecological interactions

### 5.1 *Mycorrhizal fungi in plant interactions*

Current research is addressing the question whether mycorrhizal fungi influence the outcome of plant competitive interactions. This is particularly important, not only to understand the interactions of plants in natural ecosystems, but also the effects of global change, such as the dispersal of invasive aliens on ecosystem structure and function (Dawson et al. 2012). The mycorrhizal status has a great impact on plant competition. Experiments with AM plants showed that usually plant size decreases without mycorrhizal association. This is based on the potential inability of mycorrhiza forming plants to effective use soil resources in the absence of mycorrhizal colonization (Facelli et al. 1999, van der Heijden et al. 2003). In mycorrhizal association the level of interplant competition increases considerably with enhanced use of available soil volume.

Also mycorrhizal types EM or AM might differently modify plant interactions. Aerts (2002) suggested a theoretical model of plant competition for two nutrients between plant species with different mycorrhizal types, based on Tilman´s model (Tilman 1982).

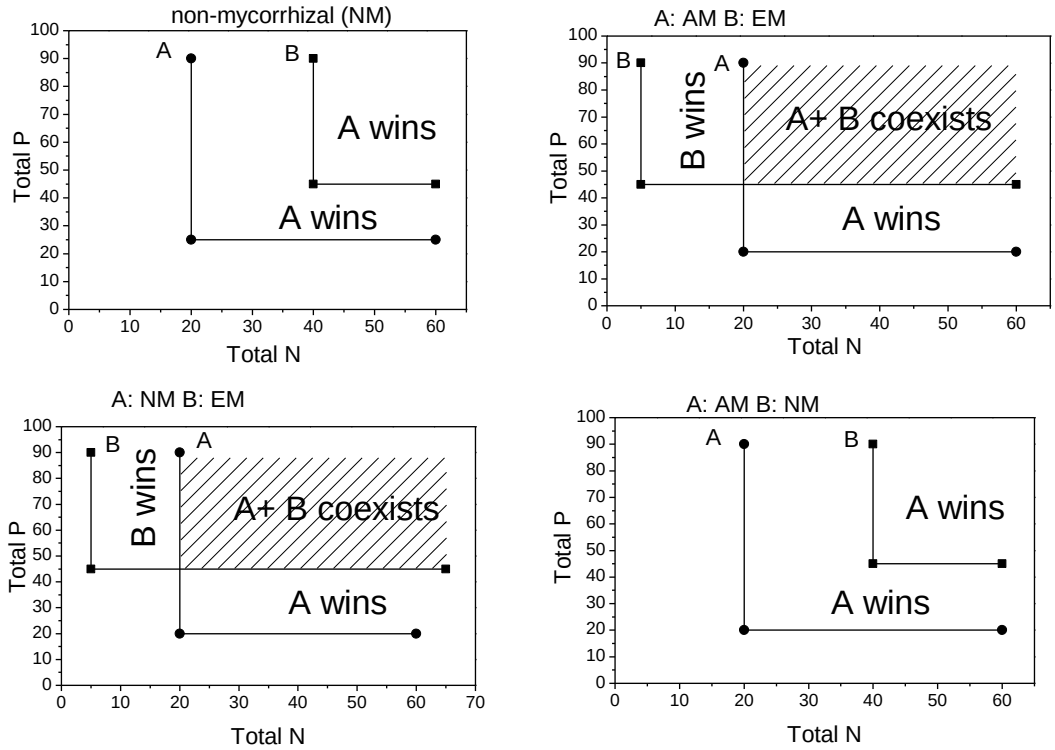


**Figure 5:** A hypothetical model to predict the effect of mycorrhizal colonization on plant coexistence in temperate forests based on Tilman´s R* model. The species that can grow on the lowest resource concentration (R*) is competitively superior to the other species. In a non-mycorrhizal (NM) situation the plant species associated with AM fungi (A) out-competes the plant species associated with EM fungi (B), because of its higher uptake capacity for both nitrogen (N) and phosphorus (P). In the mycorrhizal situation a co-existence is possible because of the increased capacity of the plant species with EM to take up N and a higher capacity of the plant associated with AM fungi to take up P. Adapted from (Aerts 2002).

The model is based on the assumption that nutrient utilization of two resources can lead to niche differentiation or out-competition between species. The species, which can reduce the resource to the lowest level and maintain growth, wins the competition. Co-existence is possible, when the growth of the species is differentially limited by the resources. Figure 5 demonstrates the suggested role of mycorrhizal type on plant interactions. In the absence of mycorrhizal colonisation, plant species associated with AM fungi is predicted to have a competitive advantage over plant species associated with EM fungi due to their presumably faster N and P uptake (Schulz et al. 2011, Stadler et al. 1993). The mycorrhizal colonization changes the situation. The suggested higher uptake capacity of EM for N, and AM for P leds to an increased P status of plant associated with AM fungi and increased N status of plant associated with EM fungi. According to Tilman´s model, both species can co-exist under these conditions.

The shift between co-existence and competition however varies with the total amount of the nutrient acquisition. Moreover, a number of influencing factors, such as plant species identity and species assemblages of root colonizing fungi have a great influence on plant performance (van der Heijden et al. 2003, van der Heijden et al. 1998). In an experiment with the AM forming plant species *Hieracium pilosella*, *Bromus erectus*, and *Festuca ovina* and four AM fungi, van der Heiden et al. (1998) demonstrated that plant species differ in their dependency on AM. This was reflected by the differing growth response of plant species on mycorrhizal colonisation, as well as by different effects of both AM species identity and species assemblages on several plant growth variables. Mycorrhizal diversity might also acts as an insurance to sustain plant productivity under changing environmental conditions. In a greenhouse experiment (Wagg et al. 2011) demonstrated that under nutrient limited conditions high number of AM mycorrhizal species relaxed the interspecific competition by reducing the growth suppression of the competitively weaker plant species. In nutrient-rich systems, the mixture of four AM fungal species was equally beneficial for the plant productivity as the most beneficial mycorrhizal fungal species in low nutrient system (Wagg et al. 2011).

### 5.2 *Mycorrhizal networks*

Both AM and EM fungi form simultaneous associations with trees of one or more taxa (Bent et al. 2011). These mycorrhizal networks (MN) are able to transport nutrients and carbon between tree individuals, and create facilitative effects of nutrient and water partitioning. This

might be particularly important to relax the aboveground competition between mature plants and seedlings (Teste & Simard 2008). In a review of 60 cases, in which seedlings and larger plants were grown together, van der Heijden and Horton (2009) demonstrated that MN promoted seedling growth in 48% of the cases, whereas in 27% cases the effect of MN was neutral and in 25% cases negative (van der Heijden & Horton 2009). Generally, plants with EM benefitted from the MN, while the effects of AM association varied (van der Heijden & Horton 2009). The type of mycorrhizal association might be particularly important, thus MN can strongly affect the growth and survival of plant species excluded from the prevailing MN (Booth 2004) and finally enhance the dominance of plants with one mycorrhizal type over another (McGuire 2007).

### 5.3 *Trophic interactions with soil fauna*

Mycorrhiza serve as an important channel of plant mediated carbon to soil food web (Pollierer et al. 2007). The use of $^{13}CO_2$ gas labelling has currently confirmed C from recent photoassimilates as the most important C source of soil animals. Besides living or dead roots and root exudates, EM hyphae presumably contribute in a considerable manner to the nutrition of soil animals (Landeweert et al. 2001).

Spore findings of EM in guts of arthropod fungivores (mites, springtails, millipedes, beetles, fly larvae) and predators (centipedes) suggest that diverse soil animals feed on mycorrhiza and serve the spore dispersal of belowground fruiting species (Lilleskov & Bruns 2005). Feeding experiments with axenic fungal cultures have shown that soil animal species feed selectively on distinct fungal species (Hiol et al. 1994, Scheu & Simmerling 2004). However, due to differences in EM metabolism in the symbiotic stage and the large variety of EM species in natural communities (Lang & Polle 2011), feeding choice experiments can hardly reflect animal behaviour under natural conditions. Currently, no firm proof for the mycorrhizal structures as primary diet of certain soil animals exists (Högberg et al. 2010, Pollierer et al. 2007). Furthermore other kinds of interaction, such as interactions between mycorrhizal and saprophytic fungi (Cairney & Meharg 2002, Mougel et al. 2006) or soil bacteria (Frey-Klett et al. 2007) occur. However, they are not considered in this thesis, since the research here focused on interactions with soil fauna.

# 6 Ecological characteristics of beech (*Fagus sylvatica*) and ash (*Fraxinus excelsior*)

**Figure 6:** Beech (*Fagus sylvatica*)

Beech (*Fagus sylvatica*) is under natural conditions, with few exceptions, the dominating tree species in monocultures and mixed forest stands in Central Europe. Currently, beech comprises approximately 30% of the forest area in Germany (www. bundeswaldinventur.de).

Beech develops a typical heart root system with a markedly dense fine root fraction. The rooting depth depends strongly on the aeration of the soil. The main rooting direction is downwards, at an angle of ca. 45° (Rust & Savill 2000). The beech roots divide into fine rootlets and end in fine tips. Beech roots do not extend very far and the rooting is very intensive, especially in the top 5-10 cm (Rust & Savill 2000).

**Figure 7:** Beech leaf

Beech has wide habitat amplitude. It favours habitats with Atlantic climatic characters; moist, warm conditions, relatively warm winters and high precipitation, avoids stagnant moisture and too dry soils (Ellenberg & Leuschner 2010). Especially in a young age beech is sensitive to winter frost, late frost, heat and dryness (Geßler et al. 2007). The best growth is reached in moist and nutrient rich soils.

Ash (*Fraxinus excelsior*) is a common deciduous tree species in entire Europe, with the exception of north Scandinavia and south Spain. In Germany ash is found in all areas, in the low mountain range up to 800 m and in the Alps up to 1350 m (Ellenberg & Leuschner 2010).

Ash root systems are superficial but far reaching. It has a strong horizontal root system that sends laterals vertically down-wards. The superficial rooting is very intensive and dominates the upper 0-5 cm of the soil profile. The fine roots usually end sud-denly and blunty (Rust and Savill, 2000). With its root system ash is able to grow in compact and wet soils. In wet soil the fine roots grow preferentially in the patches with higher aeration.

Ash has a high demand on soil nutrient richness and humidity, though it occurs on a wide range of soil types, particularly at basic (pH 6-7), calcarous soils. The opti-mal growth is reached only on fertile, pH-neutral, deep, moist and freely draining soils (Kerr & Cahalan 2004).

**Figure 8:** Ash (*Fraxinus excelsior*)

**Figure 9:** Ash leaf

Its occurrence on sites which are marginal or less optimal is probably due to competition with other species on better sites, frequently mediated by forest management.

Beech and ash differ in their light demands. Especially in the juvenile stage, beech tolerates shade (Emborg 1998). Its growth in shade is plagiotrophic, indicating a horizontal light-foraging strategy (Petritan 2009). The growth of ash follows a cap-growth strategy, characterised by a constant growth response to increasing light and an inability to strongly reduce the growth rate in deep shade (Emborg 1998, Petritan 2009).

In mixed stands of ash and beech the relative strength of plant interactions varies with stand development. The distribution of ash saplings is mainly influenced by the competition for light, whereas the interspecific competition for water strongly limits the growth and survival of beech (Rust & Savill 2000). Compared with beech, ash is more tolerant to drought (Rust & Savill 2000). In contrast, beech saplings tolerate shade better than ash. In mixed stands ash dominates the canopy in the first (~60) years of growth due to its faster growth. In the biostatic phase of the forest development, the dominance shifts from ash to beech. From now on beech is able to shade ash and reduce its biomass productivity (Emborg 1998). Interesingly, in age between 100 and 200 years, the upper 20 cm of the soil is apparently dominated by ash roots (Jacob et al. 2012, Rust & Savill 2000).

It is well known that different tree species require different N and P concentrations in their leaves for optimal functioning and growth (Güsewell 2004). According to foliar threshold values, normal ranges of foliar N and P for beech are 18.7-23.2 mg $g^{-1}$ and 1.2- 1.9 mg $g^{-1}$ respectively (Mellert & Göttlein 2012). Corresponding values for ash are 23-28 mg N $g^{-1}$ dwt and 1.4-1.6 mg P $g^{-1}$dwt, respectively (Kopinga & van den Burg 1995). The leaf N and P concentrations have been suggested to reflect the availability of these nutrients in certain habitats and therefore used as an index of the nutrition (Berger & Glatzel 2001, Güsewell 2004). At sufficient nutrient availability the N and P threshold values are reflected by leaf N/P ratios of 12.2-15.6 for beech, and 16.4-17.5 for ash. Contradictory results about the preference for mineral N forms in ash and beech exist. Geßler et al. (1998) reported a preference of beech for $NH_4^+$ both in the field and under laboratory conditions, whereas others have demonstrated that beech trees to prefer $NO_3^-$ over $NH_4^+$ (Dannenmann et al. 2009, Schulz et al. 2011, Simon et al. 2011). Reports for ash suggest a moderate preference for $NH_4^+$ over $NO_3^-$ (Stadler et al. 1993), preference for $NO_3^-$ over $NH_4^+$ (Schulz et al. 2011), or no preference for the given N forms (Jacob et al. unpublished). These observations however do not consider the role of mycorrhizal colonization, whose changes may partially explain the variability of the results within one tree species.

## 7. Objectives

This thesis focuses on the question how mycorrhizal fungi relate to plant competitive interactions and multitrophic interactions in the soil food web.

The following research questions guided the present thesis:

- Ash and beech often co-occure in mixed temperate forests. Does plant competition for nutrients relate to root colonizing mycorrhizal fungi? Concerning this research question, the nutrient uptake of beech and ash seedlings was studied in conspecific and heterospecific mixtures. The path of inorganic phosphorus, and nitrogen were studied with radioactive ($^{32}P$, $^{33}P$) and stable ($^{15}N$) tracers (Chapter 2 and 3).

- Interspecific interactions between ash and beech have often been compared without considering the associated mycorrhizal fungi on tree interactions. Which role do different EM fungi have in the nutrient acquisition of beech? In order to discover species differences of EM fungi, their contribution on nutrient acquisition of beech was studied. Uptake of mineral P (Chapter 2) and mineral N (Chapter 3) was studied in non-mycorrhizal root tips as well as in root tips colonized with different EM species of beech and compared with the nutrient accumulation in fine roots of ash.

- The effects of tree species interactions on simultaneous N and P accumulation are barely examined. What relationship do N and P accumulation of ash and beech have in species interactions, and how do N and P accumulation relate in different EM species?

- Mycorrhizal fungi are presumably an important channel of plant C into soil food web. We studied the paths of plant C via mycorrhizal fungi to soil animal food web and soil-derived N to plants. For this reason, natural regeneration of beech from Hainich National Park with intact mycorrhizal and soil animal community was grown in mesocosms for one growth season and labelled with $^{13}CO_2$ and $^{15}NO_3{}^{15}NH_4$ (Chapter 4).

The analyses of the impact of mycorrhizal fungi on nutrient and C fluxes between trees and soil food web will provide basic information that so far has been lacking. In addition, this thesis may give information about factors, which influence the co-occurrence of tree species with differing mycorrhizal types.

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# Chapter 2

# THE COMPETITION FOR PHOSPHORUS BETWEEN BEECH (*FAGUS SYLVATICA*) AND ASH (*FRAXINUS EXCELSIOR*)

## 2.1 Introduction

The role of mycorrhiza for plant productivity and competition for nutrients attracts growing interest in ecological research. It is well known that mycorrhiza has a great impact on plant nutrient acquisition, especially in nutrient limited conditions (Scheublin et al. 2007, Smith & Read 2008, Smith et al. 2003, Treseder 2004). Phosphorus (P) is essential for plants as a structural element with numerous functions in metabolism and growth. It has been suggested that plant competition for phosphorus depends mainly on its availability in soil (Allcock 2002). Due to its high reactivity with soil particles (absorption), positive ions (precipitation) and P uptake by microorganisms (immobilisation), the main part of P in soil is not available for plant uptake (White & Hammond 2008). This makes it beside nitrogen (N) to the most forest growth limiting nutrient worldwide (Abel et al. 2002, Rausch & Bucher 2002, Schachtman et al. 1998). In terrestrial ecosystems P limitation is widespread (Wardle et al. 2004). In European forest ecosystems, especially the chemical composition of the foliage of beech (*Fagus sylvatica*) indicates P deficit (de Vries et al. 2003).

In plants forming mycorrhizal symbiosis, the competition for nutrients is mainly carried out by mycorrhizal fungi (Facelli & Facelli 2002, Hodge 2004, Richardson et al. 2009, van der Heijden & Horton 2009). Tree species in temperate forests form different mycorrhizal life forms, whereof ectomycorrhiza (EM) and arbuscular mycorrhiza (AM) are the most abundant. Both AM and EM types have been reported to take up phosphorus (reviewed in Plassard and Dell 2010). Still, EM has been usually associated with uptake of other nutrients, especially N (Corrêa & Martins- Loução 2001). Because most tree species associate only with one type of mycorrhiza, distinct mycorrhizal communities occur in mixed forests. These in turn might differ in their access to P. Since the preservation and extension of mixed forest stands has become an important aspect of sustainable forest management worldwide, knowledge about competition of tree species with differing mycorrhizal types for P is of great relevance.

Linkage of trees to mycorrhizal network has a strong impact on plant growth as well as on inter- and intraspecific competition (Selosse et al. 2006, Teste & Simard 2008). For example, Teste and Simard (2008) showed that an access to mycorrhizal network increased the seedling survival in the competition experiment with Douglas-fir seedlings. In a tropical rain forest, a network of EM enhanced the survival of seedlings leading to spatial monodominance of EM forming tree species in an area usually dominated by tree species with arbuscular mycorrhiza (McGuire 2007). Hereby, the advantage of a mycorrhizal network is based on interplant

facilitation of nutrient and water exchange (Peuke & Rennenberg 2004, Selosse et al. 2006, Teste & Simard 2008).

The functional differences between plant species with different mycorrhizal strategies are of great importance for the formation of plant community structures (Cornelissen et al. 2001, van der Heijden & Horton 2009). In comparison of plants with different mycorrhizal types, plants with AM were associated with comparatively high growth ratio, high foliar N and P content and fast litter decomposition, whereas EM type trees are associated with intermediate growth ratio, lower foliar N and P contents and intermediate to slow litter decomposability (Cornelissen et al. 2001).

Despite the significance of phosphorus in tree nutrition, little research has been conducted on mycorrhizal benefits on plant P acquisition, especially in P limited systems. Controlled studies of plant-mycorrhiza interactions are often made with plants inoculated with one fungal species in an early colonization state and often hampered by low mycorrhizal colonisation. In field studies, a mixture of tree species and understory plants with diverse mycorrhizal associations hinder the study of species effects. Moreover, studies on the competition for P between tree species with different mycorrhizal types under controlled conditions are virtually inexistent.

In the present study, we aim to investigate the P competition between tree species with different mycorrhizal types in nutrient limited conditions. We performed a $^{33}P$ tracer experiment with tree saplings grown in monocultures and two-species mixtures to test the following hypotheses related to P uptake: (1) EM fungi differ in their P accumulation, (2) tree species with AM and EM have different patterns of P uptake with conspecific and heterospecific neighbour, and (3) tree species with AM association have a greater competitive effect on trees with EM association than *vice versa*;

For this purpose, we chose ash (*Fraxinus excelsior*) which form AM, and beech (*Fagus sylvatica*), which form EM, since they commonly coexist in temperate deciduous forests (Emborg 1998, Meinen et al. 2009). The plants were grown in an outdoor area and possessed natural mycorrhizal communities. Mycorrhizal colonization was confirmed before the beginning of the labelling experiment and quantified for both mycorrhizal types at the harvest. Furthermore, the EM species diversity was determined with morphological and molecular approaches.

## 2.2 Material and Methods

### 2.2.1 *Plant material*

Three year old European beech (Be, *Fagus sylvatica*, germinated June 2007) and one year old ash (As, *Fraxinus excelsior*, germinated June 2009) seedlings (seeds from Forstsaatgut-Beratungsstelle Nds. FoA Oerrel, Germany) with similar plant height were planted (01.06.2010) pair wise in three combinations: As-As, Be-Be, As-Be. Plants were grown in pots with a sand-peat mixture of 4.5 parts fine sand (ø 0.7-1.3 ø), 4.5 parts coarse sand (ø 0.4-0.8 mm) and 1 part peat in a shaded and wind protected outdoor area. Stem diameter at ground level and plant shoot height were recorded twice before (05.08.2010 and 08.11.2010) and during the harvest (26.08.2011, 29.08.2011, 02.09.2011).

In natural ecosystems plant nutrient pool is limited. In order to conduct nutrient limited conditions, plants in our experiment were grown in nutrient limited system. Plants were watered regularly with tap water (pH 7,7 - 8,4, $NH_4$ < 0.1 mg $l^{-1}$, $^{+}NO_3$, 6-10 mg $l^{-1}$, $NO_2$ < 0,04 mg $l^{-1}$, P compounds 0-0,1 mg $l^{-1}$) and fertilized for a short time from 01.10.2010 to 31.12.2010 every second day with 15 ml modified Matzners nutrient solution (Brandes 1999) with pH 3.9, containing 0.4 mM $NH_4Cl$, 0.05mM $NaSO_4$, 0.1 mM $K_2SO_4$, 0.06 mM $MgSO_4$, 0.13 mM $CaSO_4$, 0.03 mM $KH_2PO_4$ and with 0.005 mM of the following nutrients: $CuSO_4$, Fe-EDTA, $H_3BO_3$, $MnSO_4$, $NaMoO_4$ and $ZnSO_4$, respectively. In the following season (01.01.2011 -19.08.2011), the plants were exposed to rain water and if necessary irrigated with tap water to avoid drought stress. Five days before labelling (19.08.2011), the plants were moved into an experimental green house and supplied daily with 50 ml demineralised water. The growth conditions in the growth cabinet were 20°C, a 16 h photoperiod with 90-110 mmol $m^{-2}$ $s^{-1}$ photosynthetically active radiation (PAR) at plant height and 60 % air humidity.

### 2.2.2 *$^{33}P$ labelling and harvest*

Each pot was irrigated with 30 ml of the modified Matzner nutrient solution containing additionally 3.7 MBq $H_3{}^{33}PO_4$ and 4 mM $NH_4NO_3$ on day 0 to avoid acute N limitation. This

corresponded to 0.93 mg P including 6.27 ng $^{33}P$ and 3.78 mg N per pot. From now on plants were irrigated daily with 50 ml demineralised water per pot until harvest.

The number of leaves, stem lengths, stem diameters and the biomass of leaves, stem, branches, fine roots and coarse roots were determined at the harvest. Plants were divided in three groups and harvested 1, 4 and 8 days after the labelling. The roots were briefly washed with tap water to remove sand and peat and separated in fine (< 2 mm) and coarse root (> 2 mm) fractions. Aliquots of the fine roots were dried and the remaining fine roots were stored in moist tissue paper in plastic bags at 5°C for further analysis.

All plant fractions including mycorrhizal root tips, samples of vital non-mycorrhizal- and dry root tips as well as soil were dried for at least 7 d at 60° C and stored at room temperature. Dry plant fractions were homogenized with a blender (Waring Commercial Blendor, Dynamics Corporation of America, New Hartford, Connecticut, USA) and milled in a ball mill (Type MM2, Retsch, Haan, Germany).

### 2.2.3 *Autoradiography*

Autoradiography was used to qualitatively determinate the distribution of the phosphorus within ash and beech grown in monocultures and mixtures. For this purpose, one pot per treatment was used before the actual labelling experiment (05.-13.07.2011). The experimental setup was similar except following changes: 1) 1.9 MBq $H_3{}^{32}PO_4$ used instead of 3.7 MBq $H_3{}^{33}PO_4$; 2) during the experiment, plants were irrigated daily with 40 ml demineralised water; 3) plants were harvested and dried for at least 24 h at 60°C. For autoradiography images, dried fine roots and leaves were placed on paperboards, covered with transparent film (Toppits, Melitta, Minden, Germany). Subsequently, the samples were exposed on imaging plates (BASIII, Fuji Photo Film (Europe) Co., Ltd., Düsseldorf, Germany) between 30 min and 1 h depending on the amount of the radioactive decay. The plates were read out by a phosphor imager (FLA-5100, Fuji Photo Film (Europe) Co., Ltd., Düsseldorf, Germany) using an image analysing software (AIDA Image Data Analyzer software, Version 4.10.; Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

### 2.2.4 *$^{33}P$ Measurements*

Mycorrhizal root tips, fine roots and leaves (1- 5 g) were weighted, ashed at 500°C for 1 h (Heraeus M Muffle Furnace, Heraeus Instruments, Hanau, Germany) and suspended in 10 ml

of scintillation cocktail (Lumasafe$^{TM}$ plus, Lumac LSC B.V., Groningen, Netherlands). Radioactivity was determined by liquid scintillation analyzer (Tri-Carb 2800TR Counter, Perkin Elmer Life Sciences, Rodgau-Jügesheim, Germany).

Soil $^{33}P$ activity was analysed with a non-destructive $^{33}P$ analysis method, requiring no ashing and scintillation cocktail. The calibration was carried out using equivalent non-labelled samples as internal standards. For the calculation of the efficiency, internal standards were prepared by addition of 66.77 kBq $H_3{}^{33}PO_4$ in 20g dry control soil, dried and weighed in scintillation vials of 22 ml volume. Radioactivity was determined using WIZARD3 Automatic Gamma Counter (Perkin Elmer Life Sciences, Massachusetts, USA).

The $^{33}P$ activity in the plant tissues, mycorrhizal structures and soil was calculated with the equation $A$ [Bq] = (($cpm_{sample}$-$cpm_{background}$)/efficiency)/60), in which cpm = counts per minute.

The activity was corrected referring to the day of the $^{33}P$ incubation with the equation,

$$A_{(t)} = A_{(0)} * 0.5^{\frac{T_1 - T_0}{^{33}\mathrm{P}\ T_{(1/2)}}}$$

whereby $T_0$ = time of labelling $T_1$ = time of measurement and $^{33}P$ $T_{(1/2)}$ = $^{33}P$ half life (25.3 d).

### 2.2.5 *Ectomycorrhizal analysis*

The roots were examined under a stereomicroscope (Leica M205 FA, Leica Microsystems, Wetzlar, Germany). Per sample, root tips were counted until 1000 vital root tips were recorded. The numbers of vital, dead, mycorrhizal and non-mycorrhizal root tips were recorded. Live and dead root tips were separated using morphological criteria such as colour, root elasticity and the degree of cohesion of root stele and periderm (Leuschner et al. 2001). The percentage of EM colonization was calculated with the following equation:

EM [%] = (*n* mycorrhizal root tips/*n* vital root tips)*100 (Supplement table S1).

$$\text{VI [\%]} = \frac{n\ dead\ root\ tips}{n\ vital\ root\ tips} * 100$$

The EM were morphotyped by morphological characteristics according to a simplified system of Agerer (1987–2006) as described before (Druebert et al. 2009, Lang et al. 2011). All morphotypes were photographed with a digital camera (Leica DFC420 C, Leica Microsystems, Wetzlar, Germany) and the abundances were recorded. Aliquots of 10 - 20 root tips per fungal species were stored at -20 °C for molecular analysis and in 70 % EtOH at room temperature for anatomical analysis. Relative abundance (P*i*) was calculated with the equation (P*i* = n*i*/N), whereby n*i* = number of species *i* in a sample and N = number of all found mycorrhizal root tips in the sample.

### 2.2.6 *Arbuscular mycorrhizal analysis*

Ash fine roots were inspected under a stereomicroscope (Leica M205 FA, Leica Microsystems, Wetzlar, Germany) and separated according to their colour and consistence to vital and dry root tips. Aliquots were stored in 70 % EtOH at room temperature for the mycorrhizal analysis. For the determination of the arbuscular mycorrhiza (AM) colonization, the roots were stained with Lactophenol Blue (Phillips & Hayman, 1970) and stored at room temperature in 50 % glycerine until use. The roots were examined with a light microscope (Zeiss, Oberkochen, Germany) by 200 x magnification. AM colonization was recorded after the magnified intersection method of McGonigle et al (1990) on a 10 x 10 grid. Four roots per plant were analysed. The following mycorrhizal structures were recorded: vesicles, arbuscles and hyphae. The relative AM fungal colonization was calculated as follows:
AM [%] = (*n* intersections with mycorrhizal structures/ *n* intersections with root tissue)*100 (Supplement table S1).

### 2.2.7 *Molecular identification of the EM species*

DNA was extracted from 10-20 mycorrhizal root tips per sample using the innuPREP Plant DNA Kit (Analytik Jena AG, AJ Innuscreen GmbH, Jena, Germany). The ITS region was amplified with the primer pair ITS1f 5´-CTTGGTCATTTAGAGGAAGTAA -3´and ITS4 5´-TCCTCCGCTTATTGATATGC -3´ (White et al., 1990; Gardes & Bruns, 1993). PCR products were cloned in the pGEM®-T Vector (Promega, Madison, WI, USA) and transformed into electrocompetent *E. coli* cells (*E. coli* TOP10, Invitrogen, Carlsbad,

California, USA). Positive clones were sequenced with ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA) and edited with Staden Package (4.10). The fungal species were identified by comparison of the sequences with the NCBI Genbank (www.ncbi.nlm.nih.gov) and with the UNITE databases (http://www.unite.ut.ee). If the blast score was higher than 700 bits and the homology more than 97 %, the species suggested in Genbank, preferably the UNITE result was quoted. If the score was higher than 600, the homology > 95 and < 87 % and both databases suggested the same genus, the species was named as unknown species of the suggested genus (Supplement table S2).

### 2.2.8 *Fine root architecture*

Three randomly chosen fine root sections per plant were scanned on a flat-bed scanner. The pictures were analysed with WinRhizo 2005c software (Régent Instruments Inc., Québec, QC, Canada) with following settings: Number of classes: 11, filter: 0.5, sensitivity: more, 0.2 steps and dark root compatible threshold methods. Thereafter, the roots were weighed and dried at 70°C for 3 days. The dry weight was recorded. We calculated root length (RL), root surface area (SA), root volume (RV), number of root tips, forks and crossings and average root diameter (AD) as well as specific root area (SRA) and specific root surface area (SSA) for the entire root sample and for eleven root classes from 0.00 - 0.20 cm up to > 2.00 cm root diameter (Supplement table S3, and S4)

Specific root length was calculated with the equation SRL [cm $g^{-1}$ dw] = (root $length_{sample}$/root dry mass $_{sample}$), and specific surface area as SSA [cm $g^{-1}$] = ($SA_{sample}$/root dry $weight_{sample}$). The number of root tips in the sample were calculated per mg dry weight, number of forks and crossings were calculated in relation to root length (*n* $cm^{-1}$) (Supplement table S5).

### 2.2.9 *Elemental analysis*

P content of homogenised fine root and leaf samples were measured after extraction in 65 % $HNO_3$ for 12 h at 170°C according to Heinrichs et al. (1986) with an inductively coupled plasma mass spectrometer ICP-AES (Spectro Analytic Instruments, Kleve, Germany). For analysis of N and C, homogenized leaf and fine root samples were used (Supplement table

S6). EM root tips, fine root sections with AM, dead and vital non mycorrhizal fine root tips were directly weighed into tin capsules (4 x 6 mm). Samples were analysed with an isotope ratio mass spectrometer Delta C (Finnigan MAT, Bremen, Germany).

2.2.10 *Data analysis*

Statistical analyses were performed with software R 2.10.0 (Team 2008). The homogeneity of variance and normal distribution of the data were tested with Levene´s test and Shapiro-Wilk test of normality. If the preconditions of analysis of variance (ANOVA) were not met, data were log-transformed. When transformated data did not meet these requirementsKruskal-Wallist test was used to detect significant differences between the treatments As-As, Be-Be, As-Be. The pairwise comparisons were in this case calculated with Wilcoxon rank sum test.

Relations between plant nutrient element concentrations in plant tissues and plant biomass were calculated with Spearman´s rank correlation test.

We used ANOVA to analyse the effects of intra- and interspecific competition for $^{33}P$ concentrations in different plant tissues (fine roots, coarse roots, stem and branches) of beech and ash. We used species, treatment (monoculture and two species mixture) and day of harvest (1, 4 and 8) as factors and $^{33}P$ concentrations in different plant tissues and plant $^{33}P$ content as response variables. To detect significant differences between the treatments, we pooled the data of all three harvests. The pairwise comparsions were calculated with Tukey´s HSD test.

The relative annual height growth (RAG) was calculated with the equation: RAG [%] = $(h_1 - h_0)/(h_0)*100$, whereby $h_0$ = shoot height in August 2010 and $h_1$ = shoot height in August 2011 at the time of the harvest. The figures were generated with the software Origin 8.5G (Origin Lab Corp., Northampton, USA) and R 2.10.0 (Team 2008).

The $^{33}P$ uptake rates were calculated for three sampling intervals, from day of labelling to first harvest, from first to second harvest (d1-d4) and from second to third harvest with the equation

$$v = \{[(P_2)-(P_1)]/t\}/m$$

whereby $P_1$ and $P_2$ represent the $^{33}P$ content of plants at the beginning and end of the sampling interval. $t$ is the time between the samplings and m ist the mean dry mass of the plants per time intervall. Subsequently, an average of $v$ values of both sampling intervals was calculated.

The relative competition intensity was calculated according to Cambbell and Grime (1992), with the equation:

$RCI_P = (a_{mix} - a_{mono})/a_{mono}]*100$. Hereby $a_{mix}$ and $a_{mono}$ represent the plant $^{33}P$ content [kBq plant$^{-1}$] of ash and beech saplings grown in monocultures and in mixtures.

Similarly we calculated the relative annual growth intensity

$RCI_{growth}$. Hereby $a_{mix}$ and $a_{mono}$ represent the relative annual height growth [%] of ash and beech saplings grown in monocultures and in mixtures.

## 2.3 Results

### 2.3.1 *Mycorrhizal colonization in ash and beech*

The relative abundance of mycorrhizal structures in ash fine roots in monoculture was 76 ± 3 % and in two species mixture 75 ± 4 % ($P$ = 1.0). No significant differences occurred between tree species or treatments.

For EM mycorrhizal analysis, a total of 52336 vital beech root tips were observed. The percentage of vital ectomycorrhizal (EM) root tips of beech in monoculture was 69 ± 4 % and in two species mixture with ash 71 ± 5 % ($P$ = 0.285). We recorded 21 EM morphotypes, whereof 12 EM species were identified by ITS sequence data (Supplement table S1).

### 2.3.2 *Relative abundance of EM species*

*Tomentella castanea* colonized 62% of mycorrhizal beech root tips in monoculture and 70% in the two species mixture (Tab. 2.1). The second most abundant species was *Sebacina* sp., occurring on 20% of the mycorrhizal root tips in beech monoculture and 20% in two species mixture. Other EM species colonized in average 1% or less of the mycorrhizal beech root tips. No differences occurred between the relative abundances of one fungal species in different treatments.

**Table 2.1:** Relative abundance (P*i*) of ectomycorrhizal species of *Fagus sylvatica* grown in monoculture (mono) and two species mixture with *Fraxinus excelsior* (mix)

| Species | P*i* (mono) | P*i* (mix) |
|---|---|---|
| *Paxillus involutus* | 6.1 ± 2.4a | 1.4 ± 0.3a |
| *Rhizoscyphus* sp. | 5.4 ± 2.8a | 0.0 ± 0.0a |
| *Sebacina* sp. | 19.8 ± 5.3b | 19.7 ± 4.4b |
| *Tomentella castanea* | 61.9 ± 5.8c | 69.5 ± 7.8c |
| *Tomentella badia* | 4.8 ± 1.5a | 5.0 ± 1.1a |
| Other EM species | 2.0 ± 0.8a | 4.3 ± 2.9ab |

Data indicates means (± SE). $n_{mono}$= 34, $n_{mix}$= 20. Different letters in columns indicate significant differences between fungal species with $P \leq 0.05$. Statistics was performed with ANOVA and pair wise comparisons with Tukey´s HSD test.

### 2.3.3 *$^{33}$P in soil*

Soil $^{33}$P was analysed after harvest. The mean values of beech and ash monocultures were 2541 ± 496 and 3043 ± 547 kBq pot$^{-1}$, in mixture 2537 ± 615 kBq pot$^{-1}$ , respectively. No significant differences were found between labelled pots beech and ash monocultures ($P$ < 0.138), monocultures and mixture ($P_{ash}$ < 0.101; $P_{beech}$=1.00) or between harvest days. The $^{33}$P content in control pots were beech monoculture; 2 ± 3 kBq pot$^{-1}$, ash monoculture; 3 ± 7 kBq pot$^{-1}$ and mixture 5 ± 9 kBq pot$^{-1}$ respectively.

### 2.3.4 *$^{33}$P accumulation in EM species*

One day after labelling, $^{33}$P concentration of all EM species was higher than that of background radiation. The highest concentrations of $^{33}$P were measured in EM root tips of beech and in EM rhizomorphs (Fig 2.1). Vital non-mycorrhizal root tips of beech had a significantly higher $^{33}$P concentration than dry beech root tips ($P$ = 0.014, Fig. 2.1). This result was consistent also in comparison of the treatments (Be-Be and As-Be). In contrast to this, vital and dry ash fine roots had similar $^{33}$P concentrations ($P$ = 0.999, Fig. 2.1).

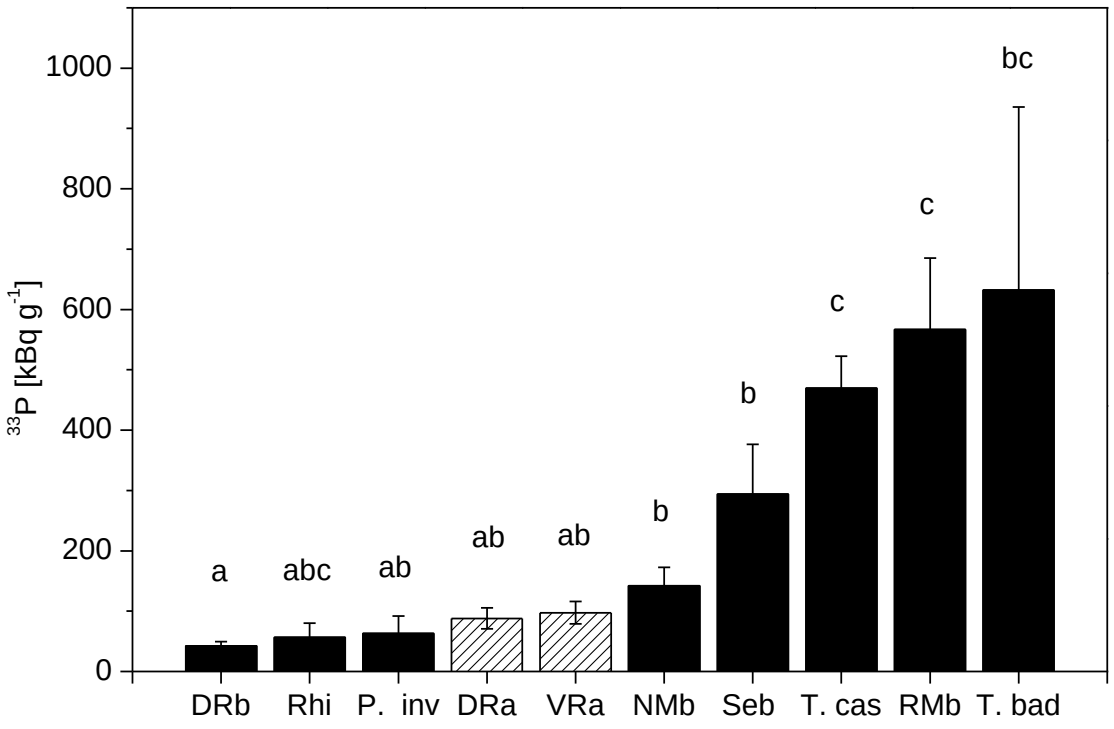



**Figure 2.1:** $^{33}$P concentration (kBq g$^{-1}$) in mycorrhizal root tips of *Paxillus involutus* (P. inv), *Rhizoscyphus* sp. (Rhi), *Sebacina* sp. (Seb), *Tomentella badia* (T. bad), *Tomentella castanea* (T. cas), Rhizomorphs (RM), non-mycorrhzal beech fine roots (NMb), vital ash root tips (VRa) as well as dry ash (DRa) and beech (DRb) fine roots labelled with $^{33}$P. Different letters indicate significant differences among plant fractions with $P \leq 0.05$. Statistics was performed with ANOVA and pair wise comparisons with Tukey´s HSD test.

For the analysis of $^{33}$P accumulation of different EM species, a total of 118 samples EM root tips of seven EM species were analyzed. Three of these species were sufficiently abundant during the time course of labelling and could therefore be used to measure $^{33}$P concentrations in mycorrhizal root tips after one, four and eight days of labelling (Fig. 2.2).

After one day labelling, all analysed EM species had reached a $^{33}$P concentration that did not increase significantly during the subsequent experiment. Only in *Sebacina* sp., $^{33}$P concentration increased in trend. *T. castanea* was highly enriched with $^{33}$P already 1 day after labelling and remained high until the end of measurement on day 8.

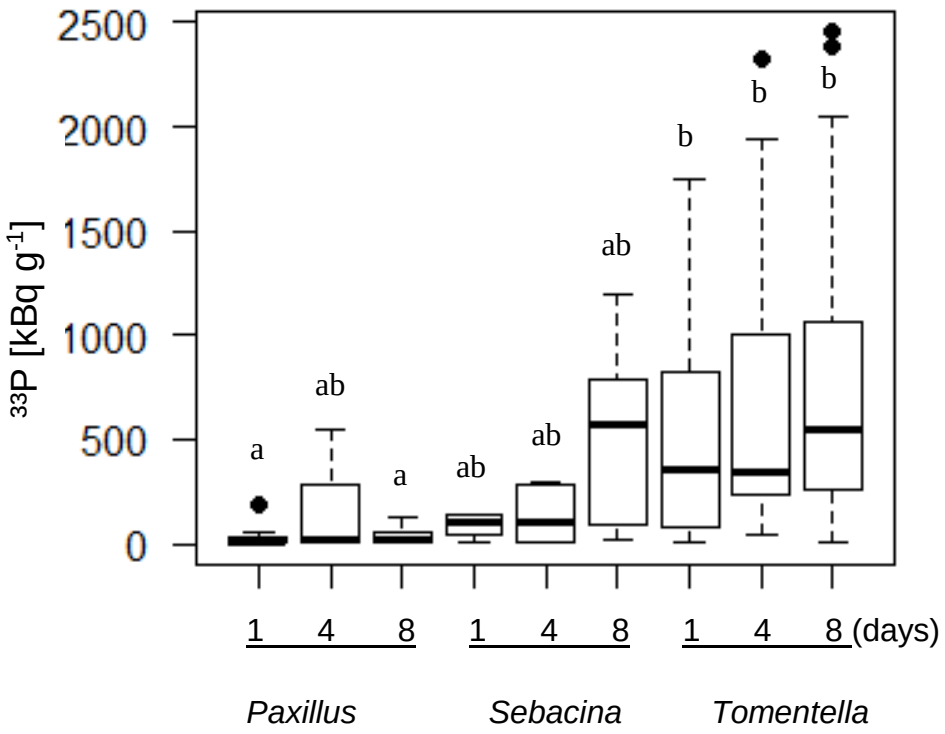



**Figure 2.2:** $^{33}P$ concentration (kBq $g^{-1}$) in mycorrhizal root tips of *Paxillus involutus* (Paxillus), *Sebacina* sp. (Sebacina) and *Tomentella castanea* (Tomentella) after 1, 4 and 8 days of incubation with $^{33}P$. Different letters indicate significant differences among plant fractions with $P < 0.05$. Outliers were excluded with Bonferroni test. Statistics: Wilcoxon rank sum test following Kruskal-Wallis test.

In contrast to these two highly P accumulative EM species, the overall $^{33}P$ concentration of *Paxillus involutus* remained low during the incubation period. Other observed EM species had rather low $^{33}P$ concentrations with the exception of *Tomentella badia*, which displayed a $^{33}P$ concentration comparable to that of *Sebacina* sp. and *T. castanea* (Fig. 2.2). Due to the low abundances of these EM species, not all time points of labelling could be measured.

### 2.3.5 *Total phosphorus (P) in fine roots and leaves*

Fine root and leaf P concentrations show that ash in mixture had lower P accumulation than in monoculture. As revealed in table 2.2, ash fine roots in monoculture had significantly higher

P concentrations than beech fine roots in monoculture ($P$ = 0.022) or beech in mixture ($P$ = 0.038). The P concentration in leaves of ash grown in monoculture was also significantly higher than in beech leaves. In contrast to this, P concentration decreased in fine roots and leaves of ash grown in mixture, resulting in P concentrations similar to those found in beech.

**Table 2.2**: Concentrations of phosphorous (P) in leaves and fine roots of beech and ash in monocultures and mixtures

| Tree species | **Treatment** | **P $_{leaves}$ [mg g$^{-1}$]** | **P $_{fine\ roots}$ [mg g$^{-1}$]** |
|---|---|---|---|
| *F. sylvatica* | mono | 0.75 ± 0.03a | 0.74 ± 0.02a |
| *F. excelsior* | mono | 0.96 ± 0.05b | 0.85 ± 0.05b |
| *F. sylvatica* | mix | 0.75 ± 0.05a | 0.75 ± 0.03a |
| *F. excelsior* | mix | 0.85 ± 0.06ab | 0.81 ± 0.03a |

Data shows means (n= 16-26 ± SE). Different letters in columns indicate significant differences among plant fractions with $P \leq 0.05$. Kruskal-Wallis test and pairwise comparisons using Wilcoxon rank sum test. Used P value adjustment method: Bonferroni.

### 2.3.6 *Phosphorus accumulation in fine roots and leaves*

To image the pattern of P distribution, autoradiography was used. Beech and ash saplings grown in monocultures and mixtures were labelled once and exposed to $^{32}$P for eight days. Figure 2.3 illustrates that after 8 days incorporation, upper leaf $^{32}$P concentrations of beech in mixture were higher than in beech grown in monoculture (Fig. 2.3 A, E). Ash leaves displayed an opposite pattern of P accumulation (Fig. 2.3 C, G). Here, the ash saplings grown in mixture with beech had lower P concentrations than those grown in monoculture after eight days labelling. $^{32}$P was unequally distributed in fine roots of both species (Fig. 2.4). Beech (Fig. 2.4 A, B) and ash (Fig. 2.4 C, D) grown in monoculture showed no differences in $^{32}$P accumulation. The autoradiograph of mixture showed, that $^{32}$P was higher beech (Fig. 2.4 E, F) than in ash (Fig. 2.4 G, H) fine roots.

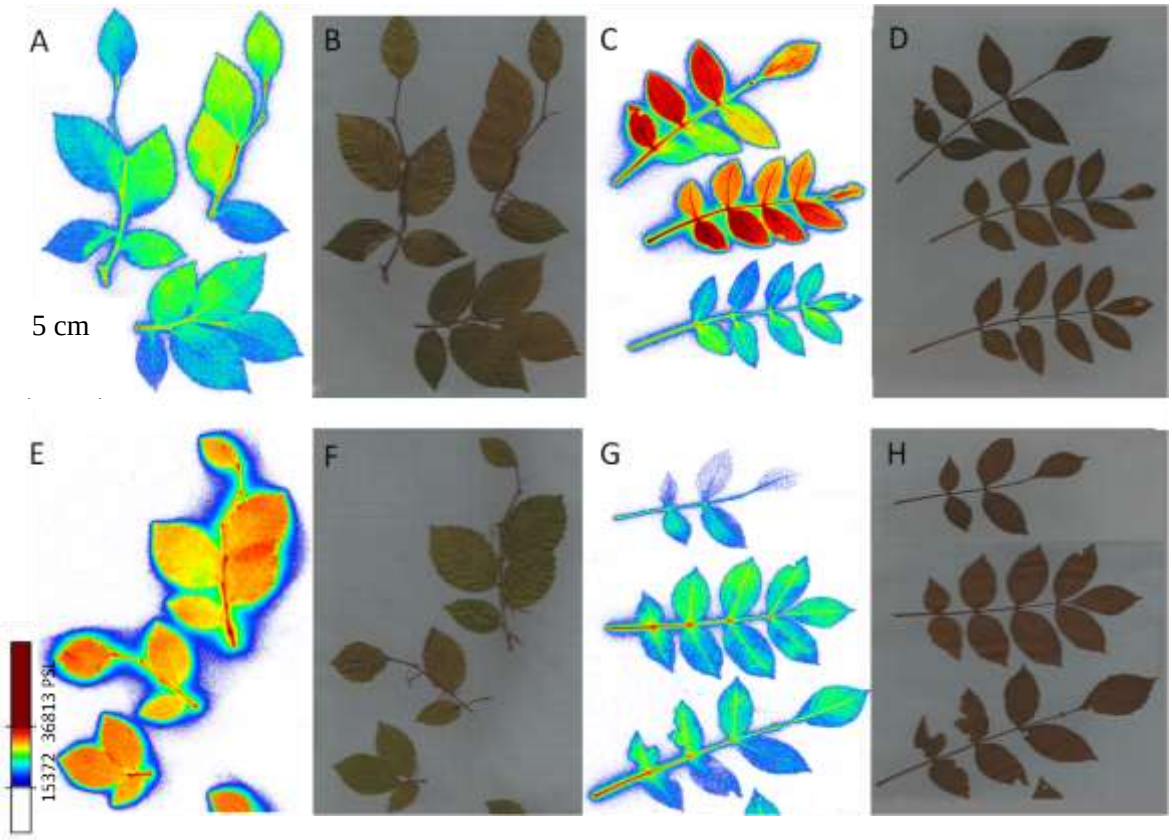



**Figure 2.3:** Representative autoradiographs and photographs of leaves of beech (A, B) and ash (C, D) grown in monocultures , as well as of beech (E, F) and ash (G, H) grown in two species mixtures after eight days labelling. The colours from blue to red indicate an increasing amount of incorporated $^{32}P$. The autoradiographs and photographs were taken in a pre-experiment under similar experimental conditions.

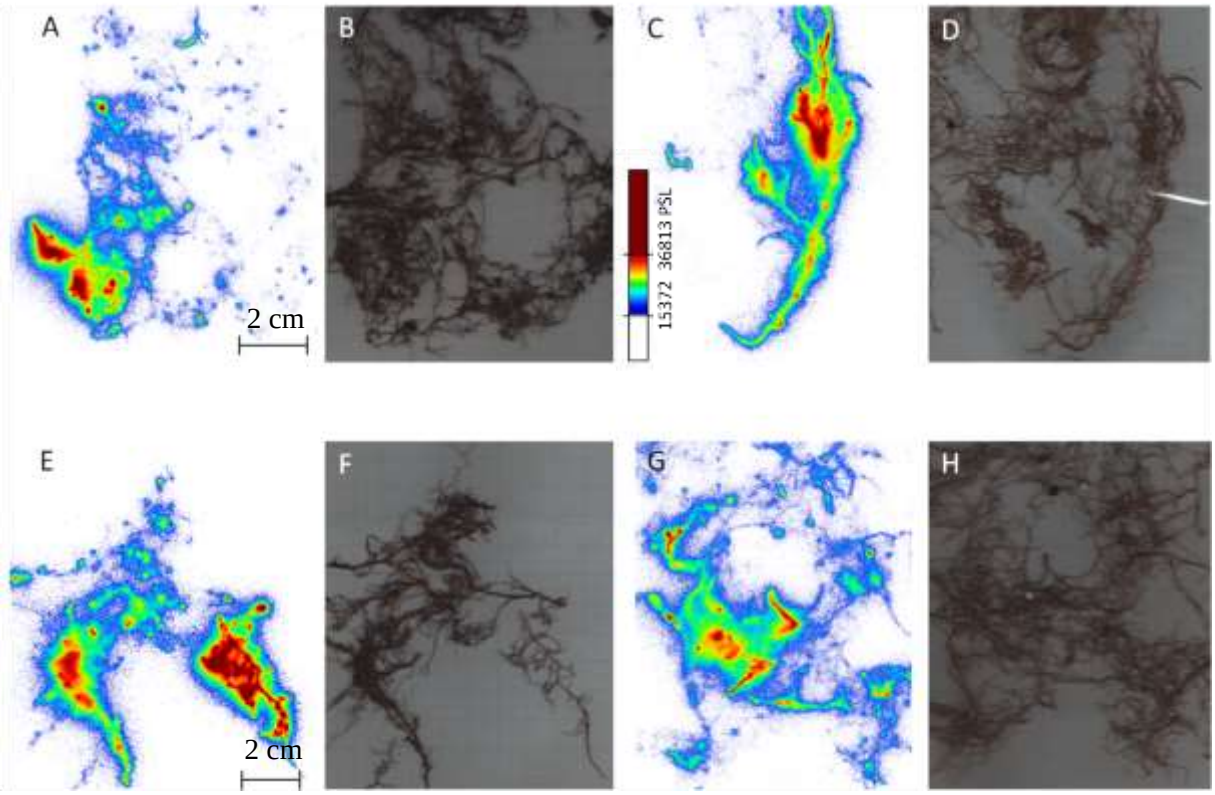
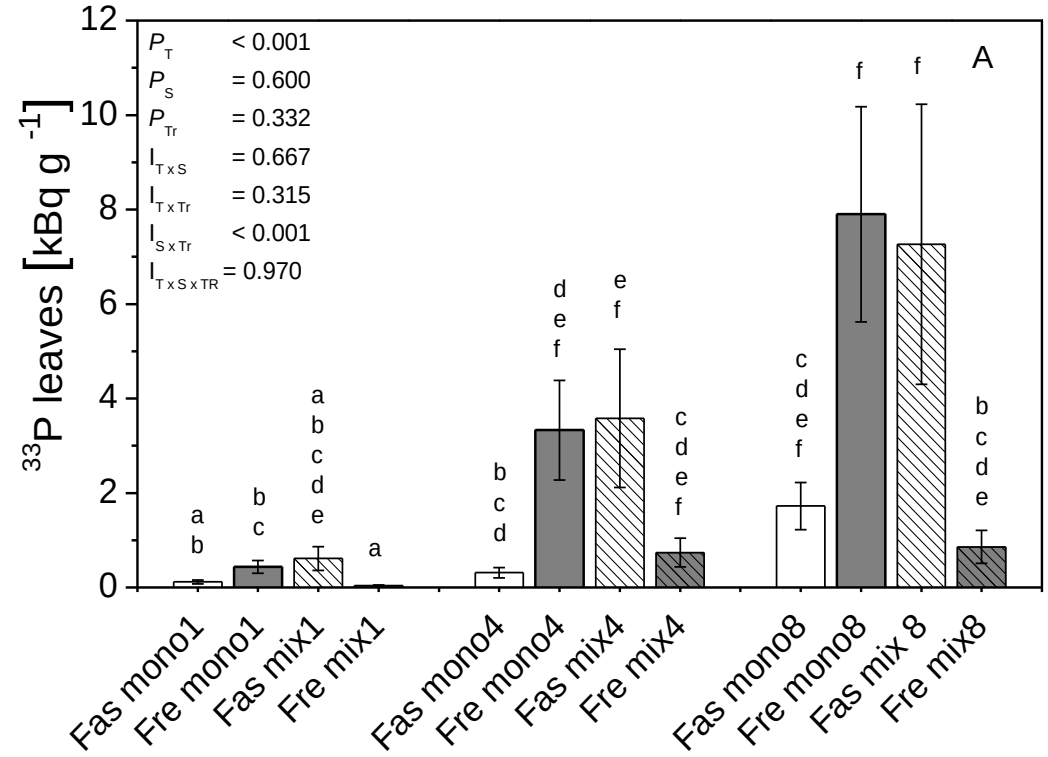
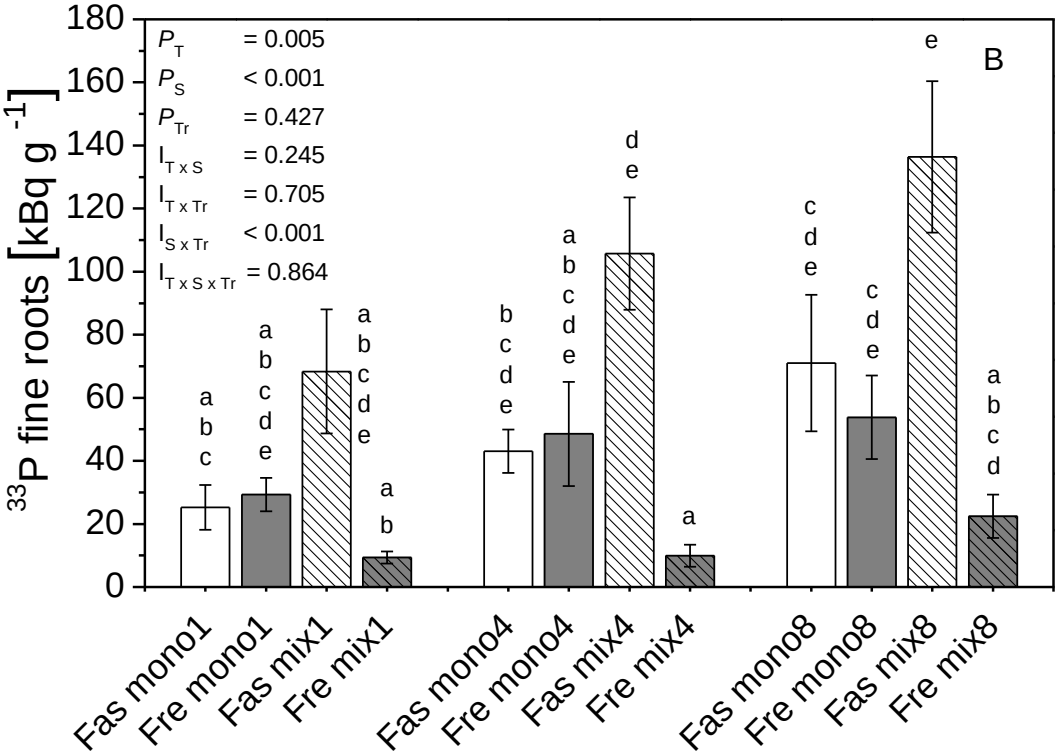



**Figure 2.4:** Representative autoradiograms and photographs of fine roots of beech (A, B) and ash (C, D) grown in monocultures , as well as of beech (E, F) and ash (G, H) grown in two species mixtures after eight days labelling. The colours from blue to red indicate an increasing amount of incorporated $^{32}P$. The autoradiographs and photographs were taken in a pre-experiment under similar experimental conditions.

Leaf and fine root $^{33}P$ concentrations as well as overall $^{33}P$ content of beech and ash increased during the experiment (Fig. 2.5). Whereas leaf $^{33}P$ concentration of ash and beech in monoculture were similar, in mixture the leaf $^{33}P$ concentrations of ash and beech differed significantly eight days after labelling ($P < 0.001$, Fig 2.5 A). The fine root $^{33}P$ concentrations of ash and beech differed also four days after labelling ($P < 0.001$). Similar differences were found overall $^{33}P$ contents of beech and ash in mixture (Fig. 2.5 B, C). Overall plant $^{33}P$ contents showed a similar pattern; however the differences were significant only four days after labelling (Fig. 2.5 C).


A
$P_T$ < 0.001
$P_S$ = 0.600
$P_{Tr}$ = 0.332
$I_{T x S}$ = 0.667
$I_{T x Tr}$ = 0.315
$I_{S x Tr}$ < 0.001
$I_{T x S x TR}$ = 0.970
$^{33}$P leaves [kBq g$^{-1}$]
Fas mono1
Fre mono1
Fas mix1
Fre mix1
Fas mono4
Fre mono4
Fas mix4
Fre mix4
Fas mono8
Fre mono8
Fas mix 8
Fre mix8


B
$P_T$ = 0.005
$P_S$ < 0.001
$P_{Tr}$ = 0.427
$I_{T x S}$ = 0.245
$I_{T x Tr}$ = 0.705
$I_{S x Tr}$ < 0.001
$I_{T x S x Tr}$ = 0.864
$^{33}$P fine roots [kBq g$^{-1}$]
Fas mono1
Fre mono1
Fas mix1
Fre mix1
Fas mono4
Fre mono4
Fas mix4
Fre mix4
Fas mono8
Fre mono8
Fas mix8
Fre mix8

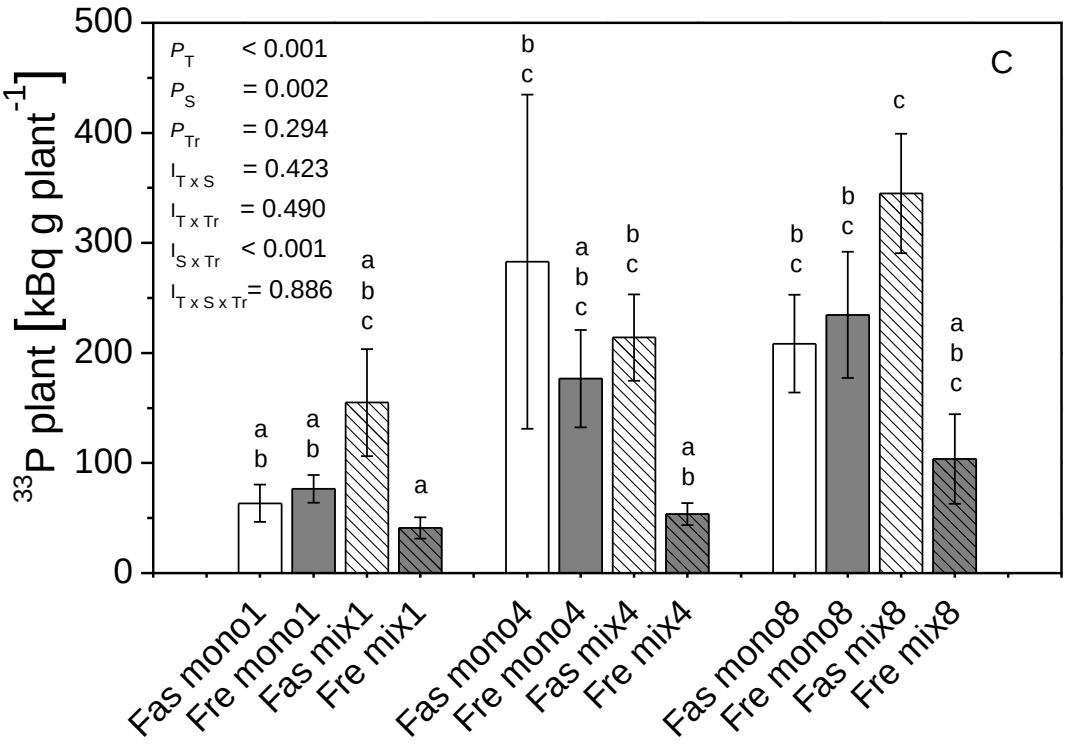



**Figure 2.5:** $^{33}$P concentration (kBq g$^{-1}$) of leaves (A) and fine roots (B) as well as overall $^{33}$P content (C) of ash and beech saplings grown in monocultures and two species mixtures after 1, 4 and 8 days labelling. Data indicates means (± SE). Different letters indicate significant differences with $P \leq 0.05$. Ti = time, S = species, T= treatment. Statistical analysis of $^{33}$P concentration [kBq g$^{-1}$] in plant tissues and overall $^{33}$P content in plant tissues [kBq dw$^{-1}$] was calculated with Tukey´s HSD test following one-way ANOVA using log transformed data.

To investigate the effect of species identity of neighbouring tree on tree phosphorus accumulation, mean $^{33}$P concentrations [kBq g$^{-1}$], $^{33}$P contents in tissues and plants across all time points were calculated (Tab. 2.3). Fine root $^{33}$P in ash decreased, whereas beech fine root $^{33}$P increased in mixture. Similar pattern was observed in leaf $^{33}$accumulation.

**Table 2.3:** $^{33}P$ concentrations [kBqg$^{-1}$] and $^{33}P$ content [kBq] of fine roots (FR), coarse roots (CR), leaves and stem, and overall $^{33}P$ content (Plant) of ash (*Fraxinus excelsior*) and beech (*Fagus sylvatica*) grown in monocultures and two species mixtures. Data indicates mean across all time points. The calculation of $^{33}P$ content is based on dry weights of tissues and total dry weights of plants

| Tree species | *F.sylvatica* | *F. excelsior* | *F.sylvatica* | *F.excelsior* | ANOVA | | |
|---|---|---|---|---|---|---|---|
| Treatmen | mono | mono | mix | mix | *SS* | *F* | *P* |
| Parameter | $^{33}P$ [kBq g$^{-1}$] | $^{33}P$ [kBq g$^{-1}$] | $^{33}P$ [kBq g$^{-1}$] | $^{33}P$ [kBq g$^{-1}$] | | | |
| FR | 49.9 ± 9.0b | 44.5 ± 6.7b | 103.5 ± 1.5c | 13.9 ± 3.5a | 7.7 | 13.8 | < 0.001 |
| CR | 0.3 ± 0.3a | 2.3 ± 0.7a | 2.0 ± 0.5a | 0.7 ± 0.2a | 1.6 | 1.6 | 0.203 |
| Stem | 1.5 ± 0.7a | 3.9 ± 1.1a | 3.2 ± 0.9a | 1.9 ± 0.8a | 3.0 | 1.9 | 0.134 |
| Leaves | 0.9 ± 0.3a | 4.1 ± 1.0b | 3.9 ± 1.2b | 0.6 ± 0.2a | 12.5 | 8.0 | < 0.001 |
| Content | $^{33}P$ [kBq] | $^{33}P$ [kBq] | $^{33}P$ [kBq] | $^{33}P$ [kBq] | | | |
| FR | 186.0 ± 50.6b | 135.8 ± 21.7b | 219.4 ± 33.3b | 55.6 ± 14.8a | 4.2 | 7.6 | < 0.001 |
| CR | 8.6 ± 3.5a | 18.5 ± 6.2a | 6.3 ± 1.0a | 7.7 ± 1.7a | 0.7 | 0.7 | 0.531 |
| Stem | 4.7 ± 1.8a | 5.0 ± 1.4a | 6.0 ± 2.2a | 7.3 ± 4.4a | 3.0 | 1.9 | 0.134 |
| Leaves | 2.0 ± 0.5abc | 6.8 ± 1.8c | 5.2 ± 1.6bc | 1.2 ± 0.3a | 9.2 | 5.6 | 0.001 |
| Plant | 188.3 ± 50.5abc | 167.1 ± 28.8c | 238.0 ± 34.3bc | 72.3 ± 17.8a | 2.9 | 4.5 | 0.006 |

Data indicates means of data from all labeling days n = 17-32 (± SE). Values of stem include stem and branches. Statistical analysis of $^{33}P$ concentration [kBq g$^{-1}$] in plant tissues and overall $^{33}P$ content in plant tissues [kBq DW$^{-1}$] were calculated with Tukey´s HSD test following ANOVA. Different letters in rows indicate significant differences with $P \leq 0.05$. df = 3, *SS* = sum of squares.

The concentration of recently accumulated $^{33}P$ was lower in fine roots of ash grown in two species mixture than in monoculture ($P$ = 0.001) and higher in beech grown in two-species mixture than in beech grown in monoculture ($P$ = 0.024). The $^{33}P$ concentrations in beech and ash leaves reflected the $^{33}P$ concentrations in fine roots. The highest plant $^{33}P$ contents were found in beech saplings grown in mixture, the lowest in ash in mixture (Tab. 2.3). The $^{33}P$ content of ash and beech saplings in mixture differed significantly ($P$ = 0.003), whereas no differences between the species were found in monocultures ($P$ = 0.945).

**Table 2.4:** Daily $^{33}P$ uptake rates of beech and ash grown in monoculture and mixture

| Species | Treatment | $^{33}P$ uptake rate [kBq $g^{-1}$] d1-d0/1 | $^{33}P$ uptake rate [kBq $g^{-1}$] d4-d1/3 | $^{33}P$ uptake rate [kBq $g^{-1}$] d8-d4/4 |
|---|---|---|---|---|
| *F.sylvatica* | mono | 4.7 ± 1.3a | 3.9 ± 2.7a | -1.9 ± 0.3b |
| *F. excelsior* | mono | 5.5 ± 1.0a | 2.8 ± 0.9ab | 0.8 ± 0.8ab |
| *F.sylvatica* | mix | 7.5 ± 3.8a | 1.4 ± 1.0ab | 2.6 ± 1.2ab |
| *F. excelsior* | mix | 1.8 ± 0.5ab | 0.2 ± 0.2ab | 0.5 ± 0.5ab |

| ANOVA | *F* | *P* |
|---|---|---|
| Species | 0.280 | 0.598 |
| Treatment | 0.052 | 0.820 |
| Time interval | 23.917 | < 0.001 *** |
| Species x Treatment | 5.225 | 0.025 * |
| Species x Time interval | 1.932 | 0.168 |
| Treatment x Time interval | 1.755 | 0.189 |
| Species x Treatment x Time interval | 0.142 | 0.708 |

Data shows means (n =6-12, ± SE). Significant differences between time intervals are marked with different Latin letters, those between mean values with different Greek letters. $P \leq 0.05$. Statistics was performed with ANOVA and pair wise comparisons with Tukey´s HSD test.

### 2.3.7 *$^{33}$P uptake rate*

Ash in monoculture had the highest average daily uptake rates, followed by beech in mixture (Tab. 2.4). The $^{33}$P uptake of beech in monoculture decreased from fourth to eigth harvest. In beech in monoculture, ash in monoculture and ash in mixture $^{33}$P uptake did not change significantly during the experiment.

### 2.3.8 *Root morphology*

The root morphological characteristics might affect the P uptake of plants. We investigated root morphological parameters and root architecture to find out whether the interspecific competition altered the root demography. Specific root length and specific root surface area of beech were significantly higher than those of ash (Tab. 2.5). Ash had a higher average root diameter than beech. The fine root architecture of beech and ash did not differ between monoculture and mixture (Table S5).

**Table 2.5:** Specific root length (SRL), specific surface area (SSA), average fine root diameter (AD) and number of root tips of ash and beech fine roots grown in monocultures and two species mixtures

| Tree species | Treat-ment | SRL [cm $g^{-1}$] | SSA [$cm^2$ $g^{-1}$] | AD [mm] | Tips [*n* $cm^{-1}$] |
|---|---|---|---|---|---|
| *F. sylvatica* | mono | 5282 ± 290a | 460 ± 24a | 2.8 ± 0.1a | 3.1 ± 0.1a |
| *F. excelsior* | mono | 3252 ± 166b | 394 ± 18a | 3.9 ± 0.1b | 1.0 ± 0.1b |
| *F. sylvatica* | mix | 5989 ± 467a | 495 ± 46a | 2.6 ± 0.1a | 1.6 ± 0.2a |
| *F. excelsior* | mix | 3592 ± 182b | 430 ± 18a | 3.8 ± 0.1b | 0.4 ± 0.1b |

Data shows means (*n* = 20-36 ± SE). ). Different letters in columns indicate significant differences among treatments with $P \leq 0.05$.Wilcoxon rank sum test following Kruskal-Wallis test.

Irrespective to the species identity of neighbouring tree, beech fine roots had a higher number of root tips, root forks and crossings, and ash a higher average fine root diameter. These results indicate that the differences in root morphology and architecture were species related and not influenced by the root competition.

### 2.3.9 *Competition intensity*

The RCI of ash was significantly smaller than zero (Tab. 2.6). In contrast to ash, RCI of beech for P did not differ significantly from zero.

**Table 2.6:** Relative competition intensity (RCI) for phosphorus in ash (*Fraxinus excelsior*) and beech (*Fagus sylvatica*). Data indicates means values of one, four and eight days after labelling and mean across all time points (1-8). The *P* values demonstrate significant differences to 0, whereby facilitation: RCI > 0 and competition RCI < 0 with P ≤ 0.05

| **Species** | **day** | **RCI** | ***P*** | |
|---|---|---|---|---|
| *F. sylvatica* | 1 | 145 ± 84 | 0.147 | |
| | 4 | -24 ± 15 | 0.171 | |
| | 8 | 65 ± 29 | 0.070 | |
| *F. excelsior* | 1 | -46 ± 16 | 0.040 | * |
| | 4 | -70 ± 6 | < 0.001 | *** |
| | 8 | -56 ± 19 | 0.032 | * |
| *F. sylvatica* | 1-8 | 62 ± 33 | 0.077 | |
| *F. excelsior* | 1-8 | -58 ± 8 | < 0.001 | *** |

Data indicates means (± SE).Statistic was calculated with t-test.

This result indicates a strong competition for P in mixture with beech compared to ash in monoculture. The results suggest that growth in species mixture with ash did not affect the P accumulation of beech, whereas ash had a competitive disadvantage.

### 2.3.10 *The effect of interpecific and intraspecific competition on plant growth*

Interspecific competition might differetially affect the plant growth than intraspecific competition. The relative growth during one growth period was faster in ash than in beech (Fig. 2.6). The growth of beech in mixture remained similar with beech in monoculture (*P* = 0.083), whereas the growth of ash decreased 55 ± 3% in mixture (*P* = 0.008). Generally, biomass was differently distributed in ash and beech (Supplement table S7), in which beech had higher aboveground biomass and ash higher root biomass. Fine root biomass however differed only between beech and ash in mixture (*P* = 0.035, Supplement table S7).

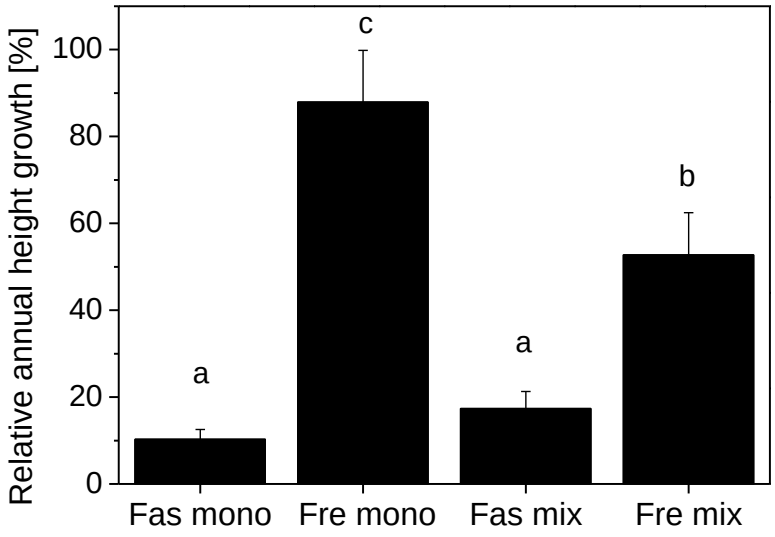



**Figure 2.6:** Relative annual height growth (%) of ash and beech grown in monocultures (mono) and in two species mixture (mix). Statistical analysis was performed with Wilcoxon rank sum test following Kruskal-Wallis test with $P \leq 0.05$.

2.3.11 *The effect of competition for phosphorus on plant growth*

In order to investigate the relationship between plant growth and relative $^{33}P$ competition intensity we calculated the relative annual growth intensity ($RCI_{growth}$) of beech and ash and compared the values with the relative $^{33}P$ competition intensity ($RCI_P$). High competition intensity of beech was related with high growth intensity (Fig. 2.7). The low competition intensity of ash linked to a low annual growth intensity. These results indicate that the P competition affected ash growth, whereas the growth intensity of beech linked with the high competition intensity for P.

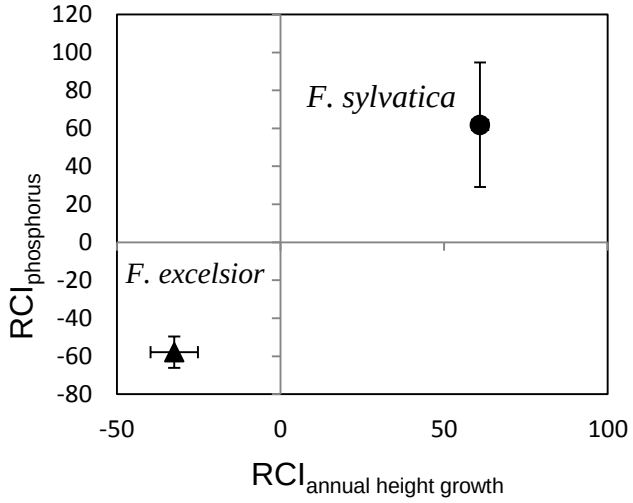



**Figure 2.7:** The relationship between relative annual P competition intensity and relative annual growth of beech and ash in nutrient limited conditions

## 2.4 Discussion

### 2.4.1 *Plant phosphorus uptake in nutrient limited conditions*

The objective of our study was the comparison of phosphorus uptake of two co-existing tree species with different mycorrhizal associations in nutrient limited system.

The importance of mycorrhizal association in plant phosphorus acquisition is particularly important in ecosystems with strong P limitation (Bücking & Heyser 2000, Bougher et al. 1990, Burgess et al. 1993). Bougher et al. (1990) showed that ectomycorrhizal infection with one of four different EM fungi (*Descolea maculata*; two isolates, *Pisolithus tinctorius*, and *Laccaria laccata*) improved plant P concentration and growth. Our experiment demonstrated, that beech colonising EM species accumulate P differently. The benefit of mycorrhizal association has been schon to decrease with the increasing level of available soil P until a point where the growth of non-mycorrhizal plants is not limited (Bougher et al. 1990). In an experiment from Burgess et al (1993), the effectiveness of 16 EM species in improving plant growth on high (12 mg $kg^{-1}$) and low (4 mg $kg^{-1}$) P levels was examined. At low soil P concentrations EM association of *Eucalyptus globulus* and *Eucalyptus diversicolor* seedlings biomass exceeded up to 13 times that of non-mycorrhizal plants (Burgess et al. 1993). We therefore presume, that the EM species in our experiment accumulated P more effieciently than they accumulate on high P levels. Also Bücking and Heyser (2000) demonstrated that P transfer from EM to *Pinus sylvestris* seedlings inoculated with *Suillus bovinus* increased in P limited conditions, whereas high external P concentration resulted in higher P content in non-mycorrhizal roots than in EM colonized roots.

### 2.4.2 *Species specific differences in phosphorus uptake of EM*

Our results are in accordance to our first hypothesis that EM species differ in their ability to take up phosphorus. The most abundant EM fungus reached high $^{33}P$ concentrations within one labelling day, whereas other EM species did not accumulate P during the labelling. In *Tomentella castanea*, the $^{33}P$ concentrations were already high after one day labelling. In *Sebacina* sp., the concentration increased constitutively during labelling period. These results indicate a rapid uptake of external P within 24h to an EM species specific level. This is in accordance with Jones et al (1991), who showed that plant P inflow rates of *Eucalyptus*

*coccifera* were 3.8 times higher with EM *Thelephora terrestris* or *L. bicolor* than that of non-mycorrhizal plants and 1.4 times higher than that of AM inoculated plants (Jones et al. 1998).

The P concentration of *Paxillus involutus* remained low during the experiment. This result indicates that *P. involutus* did not take up $H_3{}^{33}PO_4$ in a nutrient limited system. Since *P. involutus* has been demonstrated to take up P, this result might reflect the functional diversity of EM with respect to P uptake. *Paxillus involutus* might rather take up other P sources or nutrients. Bücking and Heyser (2000) showed that P content in cells of *P. involutus* and its host *Pinus sylvestris* increased with $(NH_4)_2HPO_4$ supply compared to $KH_2PO_4$ and $NaH_2PO_4$, whereas P uptake of *Suillus bovinus* was not affected by P source. One explanation for this could be an adaptation to high soil nutrient conditions, as suggested by Lilleskov et al. (2002).

The different uptake of EM species might also result from different $P_i$ transporters found in EM species. In mycorrhizal fungi, high affinity P transporters of type $P_i{:}H^+$ have been reported (Martin et al. 2008, Tatry et al. 2009). The transcripts have mainly been detected in extraradical hyphae, and their expression level is enhanced by low P concentrations in soil (Plassard & Dell 2010). Through increases in soil P*i* availability, other transporters might be activated (Tatry et al. 2009). In our experiment, *Tomentella badia* had $^{33}P$ concentrations similar to *T. castanea*. This result indicates that closely relative species might have similar phosphorus acquisition strategies.

### 2.4.3 *Phosphorus accumulation via mycorrhiza*

In accordance to our second hypothesis, trees hosting AM and EM fungi had differed in their P uptake. Our result points towards comparisons of plants inoculated with AM or EM fungi performed with tree species (Eucalyptus, Salix) that form dual mycorrhizal associations (Jones et al. 1998, van der Heijden 2001). Jones et al (1998) showed that Eucalyptus seedlings inoculated with EM (*L. bicolor* or *T. terrestris*) had higher P content, shoot and root P concentrations and higher stem mass than seedlings inoculated with AM fungi. In another experiment with *Salix repens* inoculated with AM or EM, the plant performance and phosphorus contents were measured 12, 20 and 30 weeks after inoculation (van der Heijden 2001). Here, *S. repens* inoculated with AM had faster (< 12 weeks) response in P uptake as well as in shoot and root growth, but long-term (> 7 months) plants benefitted more from EM (van der Heijden 2001). Due to the few fungal species used as inocula, the results of these two

studies might primarily reflect fungal taxon related differences in P accumulation. The measured uptake of P in AM fine root tips was remarkably low. The $^{33}P$ concentrations were comparable to those of non mycorrhizal beech fine roots. Similar fine root $^{33}P$ concentrations to those found in ash fine roots in our experiment have been reported in other plant species inoculated with the AM species *Glomus intraradices* (Nagy et al. 2005).

The differences of the mycorrhizal types in length and density of root external hyphae may result in lower concentrations of recent P in ash fine roots compared to beech. Jacobsen et al. (1992) demonstrated that the P uptake of two AM species with short external hyphae was over 25 times lower than that of AM species with long external hyphae. Compared to numerous EM species, the root external hyphae of AM are rather short (Glomus species $\leq$ 1 cm, *G. intraradices* $\leq$ 3 cm) (Agerer 1990, Jakobsen et al. 1992, Nagy et al. 2005) and do usually not form rhizomorphs (Dodd et al. 2000).

In a quantification of length of external hyphae, EM produced three to seven times more external hyphae than AM fungi. Hereby the hyphal length was highly correlated with plant P uptake and shoot weight (Jones et al. 1998). In our experiment, no quantification of external hyphae was conducted; however morphological analysis and sequence data confirmed that rhizomorphs of the most abundant EM species *T. castanea* were frequently found (Supplement table S2). The $^{33}P$ concentration in rhizomorphs had a signature similar to that of high accumulative EM root tips. Therefore, we suggest that external hyphae might have led to a competitive advantage for EM through larger space occupation and more efficient P uptake.

Early estimations by Harley and McCready (1952) showed that at low external P concentrations up to 90% of the P in EM structures might not be directly transported to beech (*F. sylvatica*). It has been suggested that high P concentration in EM structures results from permanent P uptake or serve as storage to overcome temporal P limitations (Smith & Read 2008). Though, the high P accumulation in rhizomorphs and EM mantles might have limited the available P pool for ash.

Unexpectedly, P concentrations of vital mycorrhizal ash fine roots and dead ash roots were similar. That might be explained by the morphology of dead fine roots, but need further verification.

### 2.4.4 *Total phosphorus in leaves indicates P deficiency*

The mean leaf P concentrations across all measured time points did not differ significantly between ash and beech. Based on the new critical nutrient foliar concentrations for beech (1.0 mg $g^{-1}$ P) suggested by Mellert and Göttlein (2012), beech leaf P (mono 0.8, mix 0.8 mg $g^{-1}$) concentrations indicated a P deficiency. In this study, foliar nutrient thresholds of ash were not included, but according to van der Burg´s (1985, 1990), original threshold values (1.3 mg $g^{-1}$), also ash (mono: 1.0, mix 0.9 mg $g^{-1}$) was in P deficiency. This was expected because the saplings were not fertilized in the growth period before harvest.

### 2.4.5 *$^{33}$P accumulation in leaves and fine roots*

The accumulation of recently acquired phosphorus examined with autoradiography and $^{33}$P concentrations showed that similarly to the total P concentrations, in monoculture ash leaf P accumulation was higher and increased faster than in beech. In mixture the accumulation of recently acquired P in ash clearly decreased. Similarly, the fine root $^{33}$P concentration of ash and beech resembled in monocultures, but fine roots of ash in mixture accumulated less P. These results are in accordance with our second hypothesis, that tree species with AM and EM have different patterns of P uptake with conspecific and heterospecific neighbor. Furthermore, our results indicate that ash might generally transport more or faster P to aboveground tissues than beech. There are some indications, that growth in neighbourhood of beech might affect nutrient uptake of ash. In an empirical study, nitrogen concentration of ash fine roots decreased in mixture with beech and lime, whereas P concentration of beech decreased in mixture with ash and lime (Lang & Polle 2011). However, Lang and Polle (2011) studied a tree species mixture with more than two species, which might differentially influence the nutrient acquisition of each other.

The differences in recently acquired P in ash and beech in mixture were presumably not caused by P limitation during labelling. In plant pairs, a maximum of 1% of the $^{33}$P added to the soil solution was detected (3.7 MBq $^{33}$P added per pot, in average 166 ± 60 kBq recorded in plans). However soil samples might also contain mycorrhizal hyphae.

Furthermore, results of *in vivo* and *in vitro* analysis of ash and beech saplings suggest that ash out-competes beech in water acquisition (Rust & Savill 2000). The water availability is connected with acquisition of P. The poor mobility of P in soil is further reduced by drought, whereas the uptake of N is less affected (Peuke & Rennenberg 2004). In our experiment, the plants were sufficiently watered and did not show any signs of water limitation. Beech

saplings gained more and faster the accessible P via EM. In our experiment, according to morphological and sequence data, we found numerous highly $^{33}P$ enriched rhizomorphs of EM in the soil, but no comparable AM structures.

In our experiment, the P uptake of ash did not increase in relation to biomass. Whereas ash had a higher overall root mass and higher fine root mass in mixture, both total P concentration and concentrations of recently acquired $^{33}P$ in ash tissues decreased. The comparison of monocultures and two species mixtures of ash and beech in forest showed that fine root biomass of ash increased in mixture with beech, indicating a belowground competitive superior of ash (Jacob et al. 2012). Despite greater fine root mass of ash in mixture, its P accumulation declined. Moreover, the annual growth rate of ash decreased by 55% (Fig. 2.6). These results support the conclusion that despite the higher fine root biomass, growth in mixture affected the P uptake and growth of ash saplings. In contrast to ash, beech saplings had higher $^{33}P$ concentrations in mixture but similar overall $^{33}P$ contents in both treatments. In conclusion, the effect of interspecific competition on P uptake of beech was neutral.

### 2.4.6 *Interspecific competition for phosphorus*

To our knowledge, the competition for P between beech and ash has not been reported before. In contrast to our third hypothesis, AM association did not result in a competitive advantage for ash in P utilization. The relative competition index (RCI) indicates that growth in two species mixture with beech resulted in disadvantage for ash in P uptake. Thus, the hypothesis that ash with AM has a greater competitive effect on beech with EM was rejected. The relative competition index of ash indicated strong competition for phosphorus with beech. In contrast to ash, the growth of beech in species mixture did not result in facilitative or competitive interaction with ash. In our experimental design we intended to force competition between the two tree species. Therefore, the belowground rooting area was clearly restricted.

In experiments investigating the effects of AM colonisation on intraspecific competition indicate that AM species perform best in low densities (Facelli et al. 1999). Consequently, in lower densities less root competition might occur.

## 2.5 Conclusions

In the present study, we investigated the P competition of two tree species with differing mycorrhizal strategies in a nutrient limited system.

EM fungi reveal functional diversity with respect to P uptake. The most abundant species *T. castanea* and *Sebacina* sp. emerged as high accumulative for P and reached within 24h high $^{33}P$ concentrations. The uptake of recent phosphorus in *P. involutus* remained low during the experiment, which indicates uptake of other nutrients or other ecological functions. P concentrations of rhizomorphs were similar to high accumulative EM species. Unless P in EM structures might not be directly transported to beech, the accumulation might limit the available P pool for ash and serve as a reserve that supports the host plant to overcome temporal soil phosphate delimitations.

Conspecific and heterospecific neighbor differentially affected the P uptake of ash and beech. In monocultures, the higher P accumulation of ash leaves compared to beech can be considered as taxon related characteristics. The growth in species mixture decreased ash P accumulation significantly, whereas beech P acquisition was unaffected by the species identity of the neighbour. The changes in P accumulation of ash in mixture were however not related with modification of root architecture, which indicates that mycorrhiza and not root tissue took a decisive role in P uptake.

The relative competition index of ash indicated severe competition for phosphorus with beech whereas the effect of interspecific competition on P uptake of beech was neutral.

We conclude that in nutrient limited conditions, beech with EM can effectively compete for P with ash.

## 2.6 References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Supplementary data

**Supplement Table S1:** Mycorrhizal colonization of beech (*Fagus sylvatica*; ECM) and ash (*Fraxinus excelsior*; AM) grown in monoculture and two species mixture

**Supplement Table S2:** Molecular information on ectomycorrhizal (EM) species. EM fungi were identified by ITS sequencing and sequence information was deposited in NCBI databank. If the homology was higher than 97 % and the score higher than 700 bits, the name suggested by the database, preferentially that of UNITE was quoted. If the score was higher than 600, the homology more than 95 % and both databases suggested the same genus, the species was named as unknown species of the suggested genus.

**Supplement Table S3:**Root length (RL), specific root length (SRL), specific surface area (SSA), and average root diameter (AD) of fine roots (ø ≤ 2mm) of ash and beech saplings grown in monocultures and two species mixtures

**Supplement Table S4:** The lenght (cm), area ($cm^2$), volume ($cm^3$) and number of root tips (n) of fine roots of beech (*Fagus sylvatica*) and ash (*Fraxinus excelsiot*) divided in 11 classes.

**Supplement Table S5:** Fine root architecture of beech and ash saplings grown in monoculture and in two species mixture. The number of root tips, -forks and –crossings in relation to root length

**Supplement Table S6:** Nutrient element concentrations in beech (*Fagus syvatica*) and ash (*Fraxinus excelsior*) fine roots and leafs

**Supplement Table S7:** Biometrical parameters of ash and beech grown in monoculture (mono) and two species mixture (mix)

**Supplement Table S1**: Mycorrhizal colonization of beech (*Fagus sylvatica*; EM) and ash (*Fraxinus excelsior*; AM) grown in monoculture and two species mixture

| Species | Treatment | n | % |
|---|---|---|---|
| Mycorrhizal root tips | | | |
| *F. sylvatica* | mono | 34 | 68.7 ± 4a |
| *F. excelsior* | mono | 14 | 75.8 ± 3a |
| *F. sylvatica* | mix | 20 | 70.5 ± 5a |
| *F. excelsior* | mix | 11 | 75.3 ± 4a |

Data indicates means (± SE). Different letters in columns indicate significant differences among treatments with $P \leq 0.05$. Statistics was performed with Kruskal-Wallis test. To determine ECM colonization of the root tips, in each sample 1000 vital root tips were counted. The percentage of ECM colonization was calculated as: ECM root tips/(ECM root tips + non mycorrhizal root tips)x100.

**Supplement Table S2:** Molecular information on ectomycorrhizal (EM) species. EM fungi were identified by ITS sequencing and sequence information was deposited in NCBI databank. If the homology was higher than 97 % and the score higher than 700 bits, the name suggested by the database, preferentially that of UNITE was quoted. If the score was higher than 600, the homology more than 95 % and both databases suggested the same genus, the species was named as unknown species of the suggested genus. ACC = Accession number in NCBI databank, Best BLAST match = name obtained from NCBI or UNITE

| | ACC | Length of Fragment [letters] | Best BLAST Match | Source | Strain Identity | Identities | Homology [%] | Score [bits] |
|---|---|---|---|---|---|---|---|---|
| *Hebeloma velutipes* | JX844784 | 597 | *Hebeloma velutipes* | UNITE | UDB000022 | 595/597 | 99 | 1172 |
| *Paxillus involutus* | JX844778 | 668 | *Paxillus involutus* | UNITE | UDB000754 | 642/642 | 100 | 1273 |
| *Paxillus involutus* | JX844779 | 707 | *Paxillus involutus* | UNITE | UDB000754 | 654/654 | 100 | 1296 |
| *Rhizoscyphus* sp. | JX844782 | 569 | Uncultured EM (*Rhizoscyphus*) | NCBI | HQ211588.1 | 566/569 | 99 | 1035 |
| *Sebacina* sp. 1 | JX844771 | 651 | Uncultured EM (*Sebacina*) | NCBI | HQ212339.1 | 633/653 | 97 | 1090 |
| *Sebacina* sp. 1 | JX844775 | 568 | UnculturedEM (*Sebacina*) | NCBI | HQ212339.1 | 551/569 | 97 | 948 |
| *Sebacina* sp. 1 | JX844773 | 560 | Uncultured EM (*Sebacina*) | NCBI | HQ212339.1 | 542/561 | 97 | 928 |
| *Sebacina* sp. 2 | JX844772 | 622 | Uncultured EM (*Sebacina*) | NCBI | HQ212355.1 | 604/622 | 97 | 1046 |
| *Sebacina* sp. 2 | JX844774 | 516 | Uncultured EM (*Sebacina*) | NCBI | HQ212355.1 | 502/516 | 97 | 874 |
| *Tomentella badia* | JX844776 | 704 | *Tomentella badia* | UNITE | UDB000952 | 544/546 | 99 | 1070 |
| *Tomentella castanea* | JX844764 | 619 | *Tomentella castanea* | UNITE | UDB000120 | 608/608 | 100 | 1205 |
| *Tomentella castanea* | JX844765 | 575 | *Tomentella castanea* | UNITE | UDB000120 | 575/575 | 100 | 1140 |
| *Tomentella castanea* | JX844767 | 668 | *Tomentella castanea* | UNITE | UDB000120 | 668/668 | 100 | 1324 |
| *Tomentella castanea* | JX844768 | 668 | *Tomentella castanea* | UNITE | UDB000120 | 668/668 | 100 | 1324 |
| *Tomentella castanea* | JX844769 | 668 | *Tomentella castanea* | UNITE | UDB000120 | 532/532 | 100 | 1055 |
| *Tomentella castanea* | JX844770 | 668 | *Tomentella castanea* | UNITE | UDB000120 | 637/637 | 100 | 1263 |
| *Tuber* sp. | JX844780 | 621 | Uncultured EM (*Tuber*) | NCBI | HQ204753.1 | 618/621 | 99 | 1131 |
| Uncultured EM MT10 | JX844777 | 782 | Uncultured EM fungus | NCBI | DQ233812.1 | 776/782 | 99 | 1411 |
| Unknown EM MT18 | JX844781 | 559 | *Sphaerosporella brunnea* | UNITE | UDB000994 | 345/368 | 93 | 541 |
| uncultured Helotiales | JX844783 | 466 | Uncultured EM (*Helotiales*) | NCBI | FJ475652.1 | 459/467 | 98 | 718 |

**Supplemen Table S3:** Root length (RL), specific root length (SRL), specific surface area (SSA) and average root diameter (AD)

| Tree species | treatment | SRL [cm $g^{-1}$] | SSA [$cm^2$ $g^{-1}$] | AD [cm] |
|---|---|---|---|---|
| *F. sylvatica* | mono | 5282.1 ± 290a | 459.8 ± 24.1a | 0.028 ± 0.001a |
| *F. Excelsior* | mono | 3251.6 ± 166b | 393.9 ± 18.4a | 0.039 ± 0.001b |
| *F. sylvatica* | mix | 5989.2 ± 467a | 494.6 ± 46.0a | 0.026 ± 0.001a |
| *F. Excelsior* | mix | 3591.9 ± 182b | 429.6 ± 18.3a | 0.038 ± 0.001b |

Data shows means ($n$ = 20-36 ± SE). Different letters in columns indicate significant differences among treatments with $P \leq 0.05$ (Kruskal-Wallis test and post hoc tests with Wilcoxon rank sum test).

**Supplement Table S4:** The lenght (cm), area (cm²), volume(cm³) and number of root tips (n) of fine roots of beech (*Fagus sylvatica*) and ash (*Fraxinus excelsiot*) grown in monocultures (mo) and mixtures (mi) divided in 11 classes

| Species | Treat | class 1 | class 2 | class 3 | class 4 | class 5 | class 6 | class 7 | class 8 | class 9 | class 10 | class 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Root area [cm²]** | | 0.0< - ≤ 0.2 | 0.2< - ≤ 0.4 | 0.4< - ≤ 0.6 | 0.6< - ≤ 0.8 | 0.8< - ≤ 1.0 | 1.0< - ≤ 1.2 | 1.2< - ≤ 1.4 | 1.4< - ≤ 1.6 | 1.6< - ≤ 1.8 | 1.8< - ≤ 2.0 | >2.0 |
| ***F. sylvatica*** | mo | 4.7 ±0.3a | 5.8 ±0.4a | 4.31 ±0.3ab | 1.13 ±0.1a | 0.79 ±0.1a | 0.32 ±0.1a | 0.07 ±0.02ab | 0.07 ±0.01a | 0.03 ±0.01a | 0.01 ±0.00a | 0.03 ±0.01a |
| ***F. excelsior*** | mo | 0.3 ±0.0b | 8.2 ±0.7b | 4.81 ±0.4b | 0.83 ±0.1a | 0.3 ±0.1b | 0.26 ±0.1b | 0.05 ±0.02b | 0.03 ±0.01b | 0.01 ±0.01b | 0.00 ±0.00b | 0.01 ±0.01b |
| ***F. sylvatica*** | mi | 5.2 ±0.5a | 6.8 ±0.8ab | 3.81 ±0.3a | 0.82 ±0.1a | 0.51 ±0.1ab | 0.25 ±0.1ab | 0.08 ±0.02a | 0.03 ±0.01ac | 0.03 ±0.01a | 0.01 ±0.01a | 0.03 ±0.01a |
| ***F. excelsior*** | mi | 0.4 ±0.0b | 9.8 ±1.0b | 5.65 ±0.4b | 0.76 ±0.1a | 046 ±0.1b | 0.20 ±0.1ab | 0.06 ±0.02ab | 0.02 ±0.01bc | 0.01 ±0.01b | 0.00 ±0.00ab | 0.01 ±0.00ab |
| **Root length [cm]** | | 0.0< - .≤ 0.2 | 0.2< - ≤ 0.4 | 0.4< - ≤ 0.6 | 0.6< - ≤ 0.8 | 0.8< - ≤ 1.0 | 1.0< - ≤ 1.2 | 1.2< - ≤ 1.4 | 1.4< - ≤ 1.6 | 1.6< - ≤ 1.8 | 1.8< - ≤ 2.0 | - >2.0 |
| ***F. sylvatica*** | mo | 116.2 ±6.4a | 51.1 ±3.3a | 21.5 ±1.5a | 4.11 ±0.4a | 2.28 ±0.3a | 0.74 ±0.1a | 0.13 ±0.03ab | 0.11 ±0.02a | 0.05 ±0.01a | 0.01 ±0.01a | 0.02 ±0.00a |
| ***F. excelsior*** | mo | 8.7 ±1.0b | 80.9 ±6.7b | 29.5 ±2.2b | 3.63 ±0.3a | 1.83 ±0.3a | 0.70 ±0.2a | 0.12 ±0.03b | 0.05 ±0.02b | 0.02 ±0.01b | 0.00 ±0.00b | 0.01 ±0.01b |
| ***F. sylvatica*** | mi | 127.6 ±12.3a | 61.3 ±7.9a | 19.5 ±1.7a | 3.00 ±0.4a | 1.49 ±0.2a | 0.59 ±0.1a | 0.16 ±0.03a | 0.06 ±0.01ab | 0.04 ±0.01a | 0.02 ±0.01a | 0.02 ±0.01a |
| ***F. excelsior*** | mi | 11.1 ±1.3b | 95.8 ±10.3b | 34.9 ±2.8b | 3.34 ±0.4a | 1.61 ±0.2a | 0.56 ±0.1a | 0.13 ±0.04ab | 0.04 ±0.02b | 0.02 ±0.01ab | 0.00 ±0.00ab | 0.01 ±0.00ab |
| **Root volume [cm³]** | | 0.0< - .≤ 0.2 | 0.2< - ≤ 0.4 | 0.4< - ≤ 0.6 | 0.6< - ≤ 0.8 | 0.8< - ≤ 1.0 | 1.0< - ≤ 1.2 | 1.2< - ≤ 1.4 | 1.4< - ≤ 1.6 | 1.6< - ≤ 1.8 | 1.8< - ≤ 2.0 | - >2.0 |
| ***F. sylvatica*** | mo | 0.01 ±0.0a | 0.03 ±0.0a | 0.04 ±0.0ab | 0.01 ±0.0a | 0.01 ±0.0a | 0.01 ±0.0 | 0.00 ±0.00ab | 0.00 ±0.00a | 0.00 ±0.00a | 0.00 ±0.00a | 0.00 ±0.00a |
| ***F. excelsior*** | mo | 0.00 ±0.0b | 0.06 ±0.0b | 0.05 ±0.0b | 0.01 ±0.0a | 0.01 ±0.0a | 0.01 ±0.0a | 0.00 ±0.00b | 0.00 ±0.00b | 0.00 ±0.00b | 0.00 ±0.00b | 0.00 ±0.00b |
| ***F. sylvatica*** | mi | 0.01 ±0.0a | 0.04 ±0.0ab | 0.04 ±0.0a | 0.01 ±0.0a | 0.01 ±0.0 | 0.01 ±0.0a | 0.00 ±0.00a | 0.00 ±0.00ab | 0.00 ±0.00a | 0.00 ±0.00a | 0.00 ±0.00a |
| ***F. excelsior*** | mi | 0.00 ±0.0b | 0.07 ±0.0b | 0.06 ±0.0b | 0.01 ±0.0a | 0.01 ±0.0a | 0.00 ±0.0a | 0.00 ±0.00ab | 0.00 ±0.00b | 0.00 ±0.00ab | 0.00 ±0.00ab | 0.00 ±0.00ab |
| **Root tips [n]** | | 0.0< - .≤ 0.2 | 0.2< - ≤ 0.4 | 0.4< - ≤ 0.6 | 0.6< - ≤ 0.8 | 0.8< - ≤ 1.0 | 1.0< - ≤ 1.2 | 1.2< - ≤ 1.4 | 1.4< - ≤ 1.6 | 1.6< - ≤ 1.8 | 1.8< - ≤ 2.0 | - >2.0 |
| ***F. sylvatica*** | mo | 536.5 ±30.4a | 65.3 ±9.1a | 10.5 ±1.2a | 1.93 ±0.3a | 1.03 ±0.2a | 0.27 ±0.1a | 0.10 ±0.07a | 0.00 ±0.00a | 0.00 ±0.00a | 0.03 ±0.03a | 0.00 ±0.00a |
| ***F. excelsior*** | mo | 52.4 ±5.1b | 59.1 ±5.1a | 8.53 ±0.9a | 1.28 ±0.2a | 0.72 ±0.1a | 0.28 ±0.1a | 0.08 ±0.05a | 0.00 ±0.00a | 0.03 ±0.03a | 0.00 ±0.00a | 0.00 ±0.00a |
| ***F. sylvatica*** | mi | 582.6 ±56.0a | 76.8 ±10.4a | 9.59 ±1.1a | 0.91 ±0.2 | 0.86 ±0.3a | 0.41 ±0.1a | 0.14 ±0.07a | 0.05 ±0.04a | 0.00 ±0.00a | 0.00 ±0.00a | 0.00 ±0.00a |
| ***F. excelsior*** | mi | 65.3 ±6.6b | 76.2 ±7.8a | 11.6 ±1.9a | 1.45 ±0.3a | 0.55 ±0.2a | 0.20 ±0.1a | 0.05 ±0.05a | 0.00 ±0.00a | 0.00 ±0.00a | 0.00 ±0.00a | 0.00 ±0.00a |

Data shows means ($n$ = 20-36 ± SE). Different lower-case letters indicate significant differences between the variants at a significance level $P \leq 0.05$ (Kruskal-Wallis test, post hoc test: pairwise Wilcoxon rank sum test with $P$ value adjustment with bonferroni correction method).

**Supplement Table S5:** Fine root architecture. The number of root tips, -forks and –crossings in relation to root length

| Species | Treatment | n | Tips $cm^{-1}$ | Forks $cm^{-1}$ | Crossings $cm^{-1}$ |
|---|---|---|---|---|---|
| *F. sylvatica* | mono | 30 | 3.14 ± 0.2a | 7.34 ± 0.4a | 1.50 ± 0.1a |
| *F. excelsior* | mono | 36 | 0.98 ± 0.1b | 1.88 ± 0.2b | 0.45 ± 0.1b |
| *F. sylvatica* | mix | 22 | 3.14 ± 0.3a | 7.13 ± 0.7a | 1.55 ± 0.2a |
| *F. excelsior* | mix | 20 | 1.05 ± 0.1b | 1.98 ± 0.2b | 0.44 ± 0.1b |

Data shows means ($n$ = 20-36 ± SE). ). Different letters in columns indicate significant differences among treatments with $P < 0.05$. Wilcoxon rank sum test following Kruskal-Wallis test

**Supplement Table 6:** Nutrient element concentrations in beech (*Fagus syvatica*) and ash (*Fraxinus excelsior*) fine roots and leafs

| Tree species | Treatment | Al [mg $g^{-1}$] | C [mg $g^{-1}$] | Ca [mg $g^{-1}$] | Fe [mg $g^{-1}$] | K [mg $g^{-1}$] | Mg [mg $g^{-1}$] | Mn [mg $g^{-1}$] | N [mg $g^{-1}$] | Na [mg g-$^{1}$] | P [mg $g^{-1}$] | S [mg $g^{-1}$] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fine Roots | | | | | | | | | | | | |
| *F. sylvatica* | mono | 3.78 ±0.44a | 433.4 ±6.7a | 5.08 ±0.3a | 2.95 ±0.28a | 3.24 ±0.19a | 1.48 ±0.07a | 3.78 ±0.44a | 10.09 ±0.19a | 0.66 ±0.15a | 0.73 ±0.02a | 1.48 ±0.07a |
| *F. excelsior* | mono | 0.98 ±0.16b | 432.4 ±4.8a | 3.38 ±0.1b | 0.66 ±0.08b | 9.89 ±0.62b | 3.24 ±0.14b | 0.23 ±0.16b | 7.37 ±0.21b | 2.86 ±0.23b | 0.85 ±0.03b | 3.24 ±0.14 b |
| *F. sylvatica* | mix | 5.16 ±0.77a | 429.3 ±7.9a | 4.84 ±0.2a | 3.46 ±0.44a | 3.53 ±0.67a | 1.53 ±0.09a | 5.16 ±0.77a | 9.53 ±0.35a | 0.61 ±0.03a | 0.74 ±0.03ab | 1.53 ±0.09a |
| *F. excelsior* | mix | 0.82 ±0.11b | 430.8 ±8.7a | 3.69 ±0.2b | 0.65 ±0.10b | 10.1 ±0.51b | 2.91 ±0.15b | 0.82 ±0.11b | 8.06 ±0.23b | 2.31 ±0.29 | 0.81 ±0.03ab | 2.91 ±0.15b |
| Leafs | | | | | | | | | | | | |
| *F. sylvatica* | mono | 0.13 ±0.01a | 471.5 ±8.4a | 6.28 ±0.3a | 0.15 ±0.01a | 5.05 ±0.33a | 1.83 ±0.06a | 0.59 ±0.05a | 11.96 ±0.42a | 0.17 ±0.01a | 0.75 ±0.03a | 0.82 ±0.03a |
| *F. excelsior* | mono | 0.11 ±0.01a | 435.0 ±1.7b | 11.1 ±0.7b | 0.17 ±0.01a | 16.0 ±0.77b | 4.80 ±0.27b | 0.06 ±0.00b | 11.14 ±0.36a | 0.11 ±0.02b | 0.96 ±0.05b | 1.73 ±0.10b |
| *F. sylvatica* | mix | 0.10 ±0.00a | 458.3 ±1.1c | 6.75 ±0.3ac | 0.15 ±0.01a | 5.31 ±0.82a | 2.11 ±0.15a | 0.71 ±0.09a | 11.50 ±0.65a | 0.19 ±0.02a | 0.75 ±0.05a | 0.85 ±0.07a |
| *F. excelsior* | mix | 0.12 ±0.01a | 431.1 ±1.9b | 9.22 ±0.9bc | 0.15 ±0.01a | 17.8 ±1.56b | 4.90 ±0.40b | 0.10 ±0.04b | 10.69 ±0.40a | 0.08 ±0.03b | 0.85 ±0.06ab | 1.54 ±0.15b |

Data indicates means ($n$ = 17-27 ± SE). Different letters in columns indicate significant differences among plant fractions with $P < 0.05$. Kruskal-Wallis test and Pairwise comparisons using Wilcoxon rank sum test.

**Supplement Table S7:** Biometrical parameters of ash and beech grown in monoculture (mono) and two species mixture (mix)

| Tree species | Treatment | Biomass [g plant$^{-1}$] | Leaf [g plant$^{-1}$] | Stem [g plant$^{-1}$] | Fine root [g plant$^{-1}$] | Coarse Root [g plant$^{-1}$] | R:S Ratio | CR:FR Ratio | WHD [mm] | Shoot height$^{a}$ [cm] | Shoot height$^{b}$ [cm] | RGR [%] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***F. sylvatica*** | **mono** | 20.20 ± 2ab | 2.41 ± 0.2b | 7.07 ± 0.7b | 3.73 ± 2ab | 7.52 ± 0.6a | 1.74 ± 0.4a | 2.57 ± 0.8a | 6.95 ± 0.3b | 49.07 ± 2b | 57.16 ± 2b | 1.48 ± 0.2a |
| ***F. excelsior*** | **mono** | 17.17 ± 1a | 1.54 ± 0.1a | 3.68 ± 0.3a | 3.31 ± 1ab | 8.64 ± 0.3b | 3.48 ± 0.2b | 2.93 ± 0.2b | 7.15 ± 0.2b | 19.52 ± 2a | 33.67 ± 2a | 11.02 ± 1.2c |
| ***F. sylvatica*** | **mix** | 13.90 ± 2a | 1.94 ± 0.3ab | 5.17 ± 0.8ab | 2.53 ± 1a | 4.27 ± 0.3a | 1.52 ± 0.1a | 1.83 ± 0.2a | 5.96 ± 0.3a | 43.28 ± 3b | 52.75 ± 4b | 2.67 ± 0.7a |
| ***F. excelsior*** | **mix** | 22.87 ± 2b | 1.98 ± 0.1b | 5.11 ± 0.4ab | 4.65 ± 1b | 11.12 ± 0.6c | 3.27 ± 0.2b | 2.93 ± 0.3b | 8.28 ± 0.3c | 25.42 ± 2a | 38.94 ± 2a | 5.88 ± 0.9b |

Data indicates means (± SE). Different letters in columns indicate significant differences within group with $P \leq 0.05$. Statistics were performed with Wilcoxon rank sum test following Kruskal-Wallis test. Data of relative growth rate includes control plants and was calculated with RGR [%] = (Shoot height$^{b}$-Shoot height$^{a}$)/(Shoot height$^{a}$*10)*100$^{a}$ Shoot length measured 10 months before the harvest, $^{b}$ Shoot length measured at the harvest.

# Chapter 3

# PLANT NITROGEN ACCUMULATION UNDER INTRA- AND INTERSPECIFIC COMPETITION IN RELATION TO PHOSPHORUS ACCUMULATION

## 3.1 Introduction

The benefits of mycorrhizal associations in the acquisition of the most plant growth limiting nutrients nitrogen (N) and phosphorus (P) have become important issues of scientific research (Correâ et al. 2012, Fellbaum et al., 2012, Kiers et al. 2011). The vast majority of the studies has focused on the acquisition of one nutrient element, but very little is known about the simultaneous uptake of multiple elements. In addition, the effects of mycorrhizal fungi on plant competition for multiple nutrients are nearly unknown. Therefore, the main objective of this study was to investigate the role of different mycorrhizal species on plant N and P acquisition.

N acquisition of plants presumably varies with the type of mycorrhizal association (van der Heijden et al. 2001). Especially ectomycorrhiza (EM) forming fungi have been considered to effectively increase the N status of plants (Smith and Read 2008). In an experiment with non-mycorrhizal and with EM inoculated Scotch pine (*Pinus sylvestris* L.) seedlings, root N concentration increased significantly in mycorrhizal seedlings compared to non-mycorrhizal plants (Colpaert et al. 1996). EM has been shown to be able to use both mineral N sources nitrate, $NO_3^-$ (Nygren et al. 2008) and ammonium, $NH_4^+$ (Chalot et al. 2006), most likely with a preference to $NH_4^+$, if both N forms are available (Finlay et al. 1989). Furthermore, EM fungi have been reported to use a range of organic compounds as N sources (Chalot & Brun 1998, Marmeisse et al. 2004).

Besides EM, certain tree species in temperate forests form arbuscular mycorrhizal (AM) associations. The relevance of these fungi to plant N acquisition is increasingly recognised (Fellbaum et al. 2012, Pérez-Tienda et al. 2012, Govindarajulu et al. 2005). AM fungi have been shown to take up and transport $NO_3^-$ (Tobar et al. 1994, Govindarajulu et al. 2005), $NH_4^+$ (Govindarajulu et al. 2005, Jin et al. 2005, Peréz-Tienda et al. 2012) and N from organic sources (Hawkins et al. 2000, Hodge & Fitter 2010).

Although different mycorrhizal types might impact the N and P acquisition of co-occurring tree species unequally, surprisingly little attention has been paid to this issue. According to Tilman´s (Tilman 1982) model, the nutrient utilization of two resources can lead to niche differentiation or out-competition between species (Tilman's R*). Species that can reduce the resource to the lowest level and maintain growth wins the competition. Co-existence is possible, when the growth of the species is differentially limited by the resources. The association with mycorrhizal fungi, and especially the different mycorrhizal types, modify the

response of the competing plants (Aerts, 2002). It has been suggested that in non-mycorrhizal stage, plant species associated with AM fungi have a competitive advantage over plant species associated with EM fungi. This suggestion is based on the theory that plants associated with AM are characterized by more efficient N and P uptake than plants with EM (Schulz et al. 2011, Stadler et al. 1993). The mycorrhizal colonization changes the situation. Due to the presumed higher uptake capacity of EM for N, and AM for P, the phosphorus status of ash and nitrogen status of beech increases. According to Tilman´s model, both species can co-exist under these conditions.

Until now, no experimental evidence for the differences in uptake capacities of AM and EM for N and P in competition exists. An empirical study supports the theory of Aerts (2002) for differences in N and P acquisition of mycorrhizal types (Lang & Polle 2011). Lang and Polle (2011) demonstrated that root P concentration of beech decreased and P concentration of ash increased by increasing root diversity, suggesting interspecific competition for P. In addition, N acquisition was related with EM diversity. In a mixed stand, fine root N concentration of ash decreased with increasing EM diversity of beech roots. This result indicates that EM influences the competition for N in tree species mixture (Lang & Polle 2011).

It has been speculated, that the dominance of EM tree species in temperate and boreal forests, which are characterised by a strong limitation of N availability, might rely on a more effective N acquisition of EM compared to AM (Smith and Read 2008, Rennenberg et al. 2009). However, the experimental evidence is lacking to answer the question whether EM provides an advantage in both N and P acquisition over AM symbiosis.

This study reports the nitrogen uptake of ash and beech grown in conspecific and heterospecific pairs. Since the EM species effects on plant interactions are currently not well documented, we determined N acquisition in beech root tips colonised with different EM species. It is currently also unclear whether EM species with high P accumulation, which was documented in chapter 2, also take up N more efficiently than other EM species. Therefore, the relationship of N and P accumulation in EM roo tips was studied.

For this purpose, we used beech (*Fagus sylvatica*), which forms associations with EM fungi, and ash (*Fraxinus excelsior*), which forms associations with AM fungi. Both are common tree species in Central Europe, often co-occuring in mixed forests (Ellenberg & Leuschner 2010, McKay et al. 1999). Since the developmental stage of the mycorrhizal association might strongly impact the plant response, we conducted a long term experiment to ensure the

establishment of root external hyphae and mycorrhizal colonization in plant roots. Saplings were grown in conspecific and heterospecific pairs for two growing seasons. In order to analyse N and P uptake, we labelled the saplings for one, four or eight days with a nutrient solution containing 4 mM $^{15}NO_3{}^{15}NH_4$ and 3.7 MBq $H_3{}^{33}PO_4$. Subsequently we measured $^{15}N$, $^{33}P$ and total N and P in leaves, fine roots, vital root tips (ash) as well as in mycorrhizal and non-mycorrhizal root tips (beech)

We hypothesised that (1) Nitrogen uptake of beech and ash differ in heterospecific and conspecific pairs in nutrient limited system (2) the high accumulation of P found in some EM species (Chapter 2) relate with a high accumulation for N.

## 3.2 Material and Methods

### 3.2.1 *Plant material*

The plant material and procedures have been described in chapter 2 and are therefore here only briefly reported. Beech *(Fagus sylvatica)*, and ash (*Fraxinus excelsior)* seeds were germinated in June 2007 (beech) and June 2009 (ash) (Forstsaatgut-Beratungsstelle Nds. FoA Oerrel, Germany), and grown in sand-peat mixture. In June 2010 saplings of comparable size were planted in conspecific (As-As, Be-Be) and heterospecific pairs (As-Be) in pots containing a mixture 4.5:4.5:1 fine sand (0.71-1.25 mm), coarse sand (0.4-0.8 mm) and peat.

Saplings were grown together for two vegetation periods (June 2010 to August 2011) in a shaded and wind protected outdoor area. Plants were watered regularly and fertilized every second day from 01.10.2010 to 31.12.2010 with 15 ml modified nutrient solution based on nutrient solution of Matzner et al. (1982, in Brandes 1999), containing 0.4 mM $NH_4Cl$, 0.05mM $NaSO_4$, 0.1 mM $K_2SO_4$, 0.06 mM $MgSO_4$, 0.13 mM $CaSO_4$, 0.03 mM $KH_2PO_4$, 0.005 mM $MnSO_4$ and with 0.005 mM of the following micronutrients: $H_3BO_3$, $NaMoO_4$, $ZnSO_4$, $CuSO_4$ and Fe-EDTA respectively. The plants were watered with tap water from 01.01.2011 to 19.08.2011. One week before labelling (19.08.2011), plants were moved to cabinet with constant temperature of 20°C, 16 h photoperiod, 90-110 mmol $m^{-2}$ $s^{-1}$ photosynthetically active radiation (PAR) at plant height and 60% air humidity. Plants were supplied daily with 50 ml demineralised water per pot.

### 3.2.2 *Labelling and harvest*

The saplings were labelled on day 0 with 30 ml of the modified Matzners nutrient solution per pot, containing 3.78 mg N whereof 3.36 mg $^{15}N$ and 0.93 mg P whereof 6.27 ng $^{33}P$ per pot. 1, 4 and 8 days after labelling, labelled and non-labelled plants from conspecific and heterospecific mixtures were harvested. The roots were briefly washed with tap water and separated in fine root (< 2 mm) and coarse root (> 2 mm) fractions. Subsequently, soil and plant fractions (fine roots, coarse roots, leaves, stem and branches) were weighted. Stem length, collar diameter and the number of leaves were recorded. Aliquots of fine roots were separated, put to plastic bags with moist tissue paper and stored at 5°C for mycorrhizal analyses. Subsequently fine roots, mycorrhizal samples, all other plant fractions and soil aliquots were dried at 60°C.

### 3.2.3 *$^{15}$N isotope analysis and N uptake rate*

$^{15}$N labels of fine roots, leaves, dry fine roots, vital ash fine root tips of 1. and 2. order, EM fine root tips and non-mycorrhizal beech root tips were measured. Dry plant material was homogenized with a blender (Waring Commercial Blendor, Dynamics Corporation of America, New Hartford, Connecticut, USA) and ball mill (Type MM 2, Retsch, Haan, Germany). Due to the small sample sizes, ash fine root tips, non-mycorrhizal beech fine roots and EM root tips (one EM sample containing approximately 40-100 tips) were directly used. 2 mg of the dry material were weighted into tin capsules and analyzed with a coupled system of elemental analyser (NA 1500, Carlo Erba, Mailand) and a mass spectrometer (EA NC2500 Delta Plus and EA NC1108 ConFlo III Delta C, Finnigan MAT, Bremen, Germany) at the Centre for Stable Isotope Research and Analysis, University of Göttingen. The isotopic composition was calculated as

$$\delta_{sample\ i}\ [‰] = \frac{R_{sample\ i} - R_{standard}}{R_{standard}} * 1000$$

where $R_{sample\ i}$ and $R_{standard}$ represent the stabile isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) of the sample *i* and standards. Results are shown in the δ notation in ‰ relative to the international standard PD Belemnite (PBD) for $^{13}$C and atmospheric nitrogen for $^{15}$N.

Specific N uptake rate was calculated for fine roots, leaves and root tips according to Schulz et al. (2011) with the equation:

$$\mu g\ ^{15}N\ d^{-1}\ (g\ plant\ biomass\ dw)^{-1} = [\frac{(a_1 - a_0) * N\%}{t}] * 100$$

whereby $a_1$ and $a_0$ represent the atom% $^{15}$N concentrations of the plant fractions in labelled plants and non-labelled control plants. The atom% is calculated as

$$atom\%\ ^{13}C = \frac{^{13}C}{^{13}C + ^{12}C} * 100$$

and N% is the total N concentration and *t* is the time between $^{15}$N labelling and harvest date.

The enrichment of $^{15}N$ in root tips was expressed as atom%excess

$$\mathrm{APE}_{^{15}\mathrm{N}} = (\mathrm{atom\%}_{\mathrm{labelled}} - \mathrm{atom\%}_{\mathrm{unlabelled}}) * 100$$

### 3.2.4 *$^{33}P$ Analysis*

The radiation of radioactive isotopes in mycorrhizal root tips was measured with a liquid scintillation analyzer (Tri-Carb 2800TR Counter, Perkin Elmer Life Sciences, Rodgau-Jügesheim, Germany), as described previously in chapter 2.

### 3.2.5 *Mycorrhizal analysis*

Root tips were examined under a stereomicroscope (Leica M205 FA, Leica Microsystems, Wetzlar, Germany) and divided to vital and dry tips according to their colour and consistence. The EM mycorrhizal colonization was calculated, the EM root tips morphotyped, abundances recorded and the morphotypes photographed as described previously (chapter 2). For the analysis of the relative colonization rate of ash, three lactophenole-blue stained (Schmitz et al. 1991) fine roots per sample were examined with light microscope (Axioplan with digital camera AxioCam incl. software Axiovision, Zeiss, Oberkochen, Germany) and AM colonization was calculated with the magnified intersection method described by McGonigle et al (1990).

### 3.2.6 *Plant growth*

The relative annual height growth (RAG) was calculated with the equation:

$$\mathrm{RAG}\ [\%] = \left[\frac{(h_1 - h_0)}{h_0}\right] * 100$$

whereby $h_0$ = shoot height in August 2010 and $h_1$ = shoot height in August 2011 at the time of the harvest.

### 3.2.7 *Data analysis*

The statistical analysis was performed with the software R (The R Foundation for Statistical Computing, http://www.r–project.org). Data were log-transformed to meet the assumptions of normality and variance homogeneity. We carried out analysis of variance (ANOVA) with

Tukeys HSD test. We used treatment (As-As, Be-Be, As-Be) and days of labelling (1, 4, and 8) as main effects to examine signatures of $^{15}N$ and $^{13}C$ as well as total N and C concentrations of plant tissues. If the data were unsuitable for ANOVA after transformation, we used a non-parametric Kruskal-Wallis test and pairwise comparisons with Wilcoxon test to detect significant differences.

Linear regression analyses were performed to evaluate the relationship of $^{15}N$ signatures of fine roots and leavesas well as $^{15}N$ concentrations of mycorrhizal and non-mycorrhizal fine root tips. The figures were generated with the software Origin 8.5G (Origin Lab Corp., Northampton, USA) and R 2.10.0 (Team 2008).

## 3.3 Results

### 3.3.1 *Total carbon and nitrogen in fine roots and leaves*

Total N concentrations in ash and beech leaves were similar (Tab. 3.1). According to the foliar nutrient threshold values of 19-23 mg $g^{-1}$ for beech (Mellert and Göttlein 2012) and 23-28 mg $g^{-1}$ for ash (Kopinga & van den Burg, 1995), both species were N deficient. Total N concentrations of fine roots displayed taxon related differences and were not influenced by the species identity of the neighbour.

Leaf C concentration of beech was higher than in ash, but decreased in mixture. Generally the C concentrations of fine roots were species specific. The growth in mixture increased the C concentration of EM root tips of the most abundant EM species (EM species mean). Non-mycorrhizal root tips of beech had the highest C concentrations.

**Table 3.1:** N and C concentration [mg $g^{-1}$] in leaves, fine roots (FR) as well as in dry, vital non mycorrhizal (NM) and mycorrhizal (EM) root tips of beech and dry and vital mycorrhizal root tips of ash fine roots grown in monoculture (mono) and two species mixture (mix)

| Sample | Species | N [mg $g^{-1}$] mono | N [mg $g^{-1}$] mix | C [mg $g^{-1}$] mono | C [mg $g^{-1}$] mix |
|---|---|---|---|---|---|
| leaf | *F. sylvatica* | 11.91 ± 0.4a | 11.55 ± 0.7a | 470.63 ± 8.5a | 458.81 ± 1.0c |
| | *F. excelsior* | 11.14 ± 0.4a | 10.69 ± 0.4a | 435.03 ± 1.7b | 431.07 ± 2.0b |
| FR | *F. sylvatica* | 10.06 ± 0.2b | 9.7 ± 0.3b | 428.62 ± 7.4a | 432.77 ± 8.8a |
| | *F. excelsior* | 7.37 ± 0.2a | 7.8 ± 0.2a | 432.43 ± 4.9a | 433.55 ± 7.3a |
| NM root tip | *F. sylvatica* | 14.70 ± 1.0b | 12.68 ± 0.5b | 477.82 ± 3b | 467.30 ± 18b |
| vital root tip | *F. excelsior* | 8.68 ± 0.3a | 8.92 ± 0.4a | 449.64 ± 2a | 447.21 ± 4a |
| Dry root tips | *F. sylvatica* | 14.79 ± 0.5a | 14.39 ± 0.8a | 465.20 ± 4a | 464.20 ± 5a |
| | *F. excelsior* | 15.05 ± 1.0a | 15.50 ± 0.8a | 452.65 ± 17a | 472.41 ± 3a |
| *Ectomycorrhizal samples* | | | | | |
| EM sp. mean | *F. sylvatica* | 16.20 ± 0.4a | 17.46 ± 0.9a | 439.63 ± 7a | 447.06 ± 3b |
| EM pool | *F. sylvatica* | 17.29 ± 0.4a | 16.31 ± 0.4a | 456.71 ± 4b | 445.65 ± 2b |

Data indicates means ($n_{mono}$= 13-46; $n_{mix}$= 12-23, ± SE). Different letters indicate significant differences of element concentration of a tissue between tree species in different treatments (mono–mix) with $P \leq 0.05$. Statistics were performed with Wilcoxon rank sum test following Kruskal-Wallis test.

### 3.3.2 *$^{15}$N accumulation in leaves and fine roots*

The leaf $^{15}$N accumulation of beech and ash in monocultures displayed species differences (Fig. 3.1 A, Tab. 3.2). Leaf $^{15}$N accumulation of beech was significantly lower than in ash ($P$ = 0.012). In addition, leaf $^{15}$N signature of beech did not increase remarkably during the labelling period. The signatures were however higher than in unlabelled controls ($P$ < 0.001). Leaf $^{15}$N accumulation of ash decreased in mixture compared to monoculture. This resulted in similar leaf $^{15}$N labels of ash and beech in mixture ($P$ = 0.877) at day 8. Fine root $^{15}$N accumulation of ash and beech did not differ significantly over the time (Fig. 3.1 B).

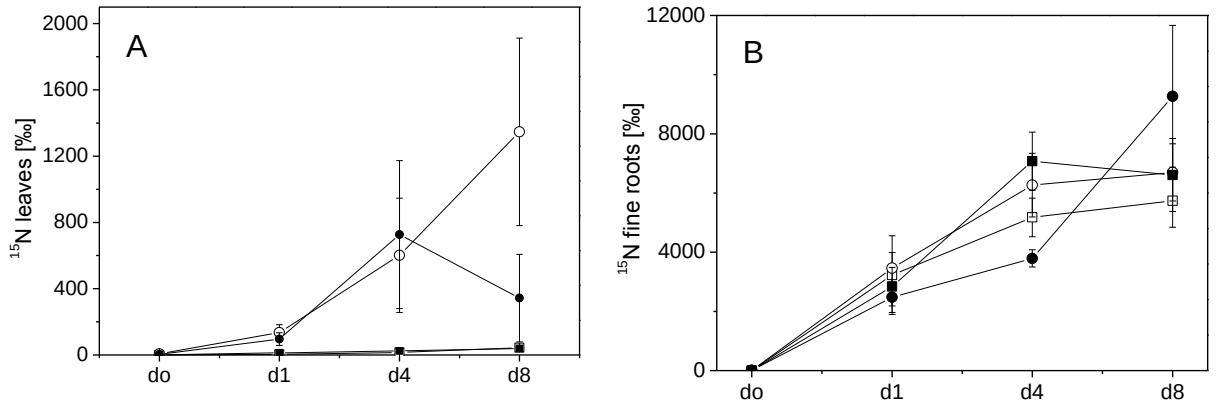



**Figure 3.1:** $\delta^{15}N$ accumulation in leaves (A) and fine roots (B) of beech (squares) and ash (dots) grown in monoculture (open symbols) and two species mixtures (filled symbols) during 8 days labelling.

**Table 3.2:** Results of ANOVA for $\delta^{15}N$ accumulation [‰] in leaves and fine roots of beech and ash grown in monoculture and two species mixtures during 8 days labelling

| ANOVA $^{15}$N leaves [‰] | *F* | *P* | |
|---|---|---|---|
| Day | 44.184 | <0.001 | *** |
| Species | 88.327 | <0.001 | *** |
| Treatment | 0.003 | 0.953 | |
| Day x Species | 2.846 | 0.042 | * |
| Day x Treatment | 0.921 | 0.434 | |
| Species x Treatment | 4.221 | 0.043 | * |
| Day x Species x Treatment | 0.394 | 0.758 | |

| ANOVA $^{15}$N fine roots [‰] | *F* | *P* | |
|---|---|---|---|
| Day | 627.444 | <0.001 | *** |
| Species | 2.634 | 0.626 | |
| Treatment | 0.240 | 0.049 | * |
| Day x Species | 2.718 | 0.506 | |
| Day x Treatment | 0.784 | 0.133 | |
| Species x Treatment | 2.298 | 0.302 | |
| Day x Species x Treatment | 0.474 | 0.701 | |

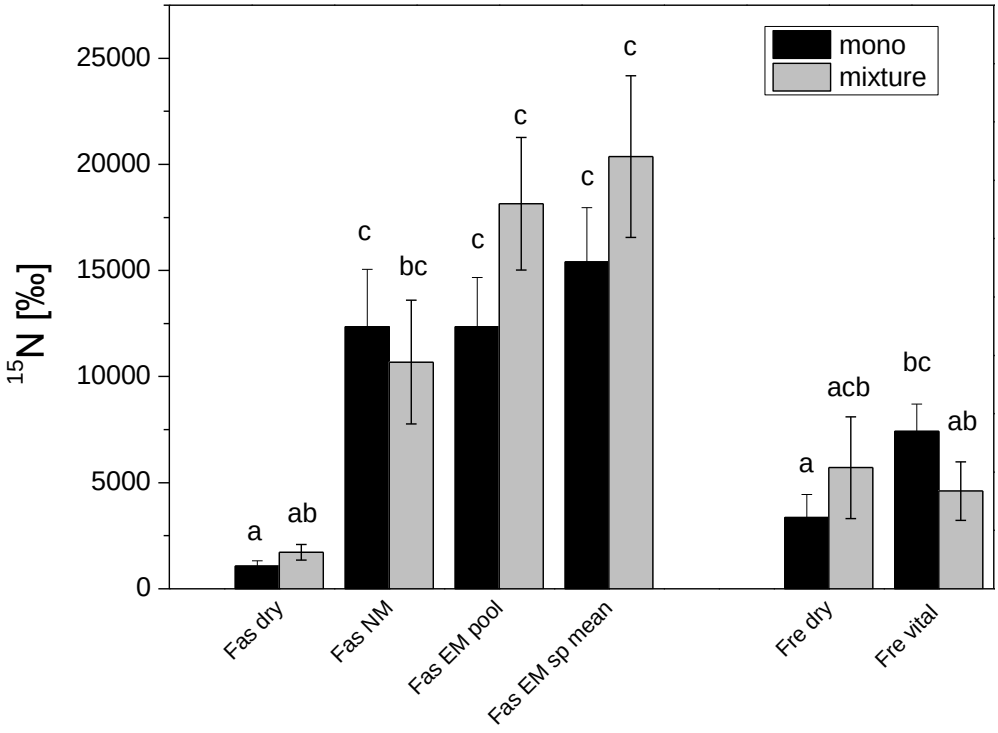
Beech fine root $^{15}$N accumulation was generally higher in mixture than in monoculture. However the accumulation of $^{15}$N in fine roots differed not significantly between species.

### 3.3.3 *$^{15}$N accumulation in fine roots tips*

In order to find out whether $^{15}$N uptake of fine root tips differs between trees grown in monoculture and mixture, we compared the mean $^{15}$N signatures of root tips (Fig. 3.2). The

highest $^{15}N$ label was found in EM root tips. In monoculture NM beech fine root tips and vital ash fine root tips accumulated $^{15}N$ similarly ($P$ = 0.219). In mixture NM beech root tips had higher fine root $^{15}N$ signatures than ash fine root tips ($P$ = 0.039).

In mycorrhizal beech root tips, the N accumulation tended to increase in mixture. In contrast, vital ash root tips and non-mycorrhizal (NM) beech root tips tended to accumulate less $^{15}N$ in mixture. The lowest $^{15}N$ signatures were recorded in dead root tips of beech. Passive absorption of $^{15}N$ was measured to some extent in dead root tips of both tree species.




| ANOVA<br>$^{15}N$ fine root tips [‰] | $F$ | $P$ | |
|---|---|---|---|
| Tissue | 21.8052 | < 0.001 | *** |
| Species | 0.814 | 0.368 | |
| Treatment | 1.502 | 0.222 | |
| Tissue x Species | 8.375 | 0.004 | ** |
| Tissue x Treatment | 0.995 | 0.396 | |
| Species x Treatment | 0.120 | 0.730 | |
| Tissue x Species x Treatment | 0.083 | 0.774 | |

**Figure 3.2:** $\delta^{15}N$ enrichment (‰) in dry, vital non mycorrhizal (NM), mycorrhizal root tips of most abundant ECM species (EM sp mean), mixture of all found EM (EM pool) of beech (Fas) and dry and vital mycorrhizal root tips of ash (Fre) fine roots grown in monoculture (mono) and two species mixture (mixture). Data indicates means (n = 46-16 ± SE). Tukeys HD test following one way ANOVA with $P \leq 0.05$.

### 3.3.4 *$^{15}$N accumulation in EM species*

In order to determine taxon related differences in N uptake in EM fungi, $^{15}$N accumulation was measured in beech root tips colonized with different EM species. Because no significant differences between monocultures and mixtures occurred, the mean value of both treatments is shown. Some taxon related differences were found in the $^{15}$N uptake of EM species (Fig. 3.3).

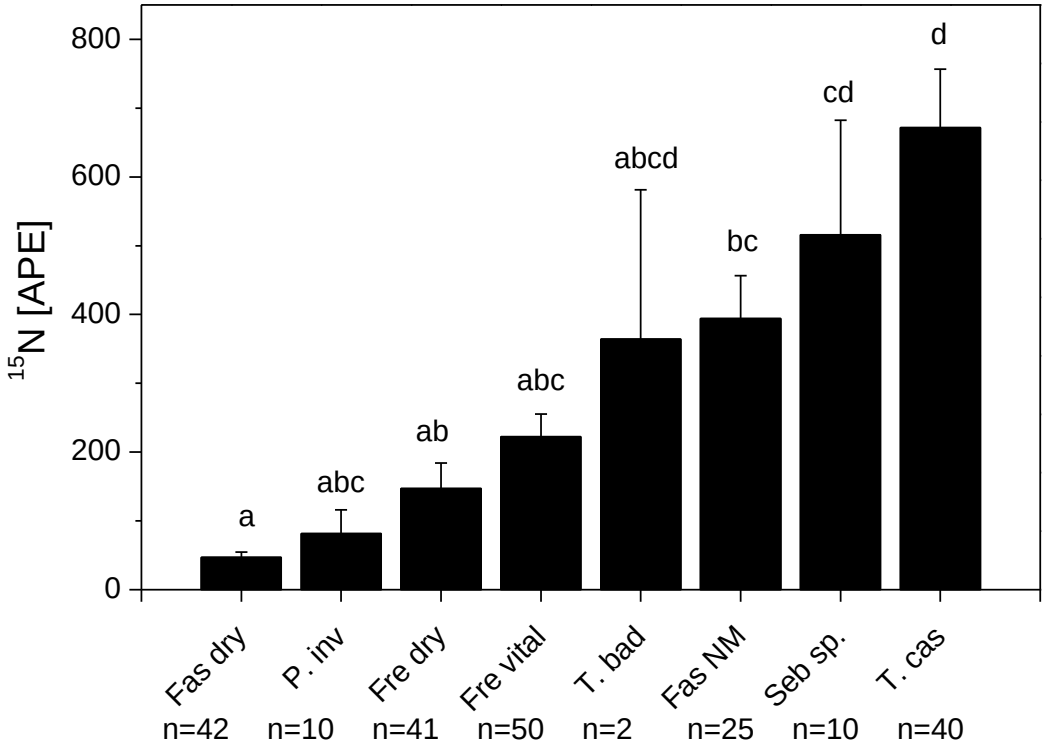



**Figure 3.3:** $^{15}$N signatures of dry root tips (dry), of ash (Fre) and beech (Fas), vital root tips of ash (vital), non-mycorrhizal root tips (NM) of beech and in ectomycorrhizal species *Paxillus involutus* (P. inv), *Tomentella castanea* (T. cas), Sebacina sp. (Seb sp) and *Tomentella badia* (T. bad). Data indicates means (± SE). Letters indicate significant differences with $P \leq 0.05$. Tukeys HD test following one way ANOVA.

The most abundant EM species *Tomentella castanea* (68 ± 3 % colonized root tips) and *Sebacina* sp. (19 ± 3 % colonized root tips) were highly enriched with $^{15}$N. In contrast, the $^{15}$N label of *Paxillus involutus* remained low (4 ± 1 % colonized root tips). Its $^{15}$N signature was similar to non-mycorrhizal root tips and dry beech roots. This result demonstrates that whereas other EM sepcies accumulated N effectively, *P. involutus* barely accumulated nitrogen.

Despite the differences between $^{15}N$ singatures of EM species, total N contents of EM species were relatively similar (Fig. 3.4). This result suggests that the higher $^{15}N$ signatures found in *Tomentella* spp. and *Sebacina* sp. did not result from a generally higher N content of fungal structures.

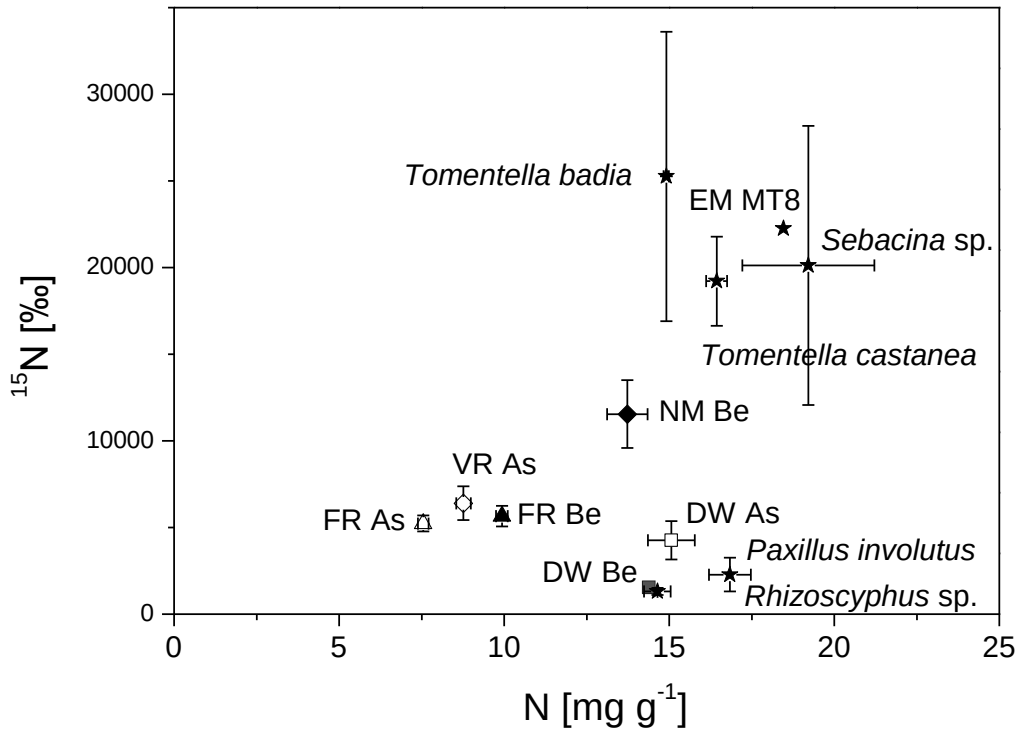
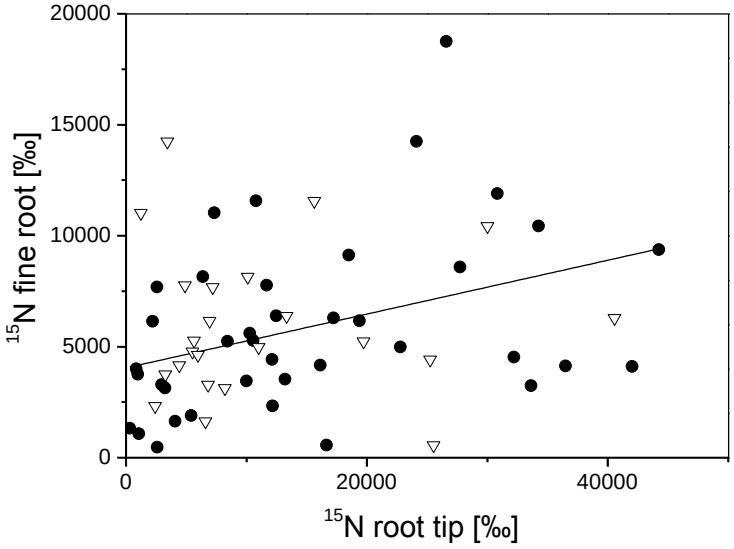



**Figure 3.4:** The relationship of $^{15}N$ accumulation and total N concentration in ash (open symbols) and beech (filled symbols) fine roots (triangles), non-mycorrhizal root tips of beech (square), vital root tips of ash (square), dry root tips (rectangle) and root tips colonized with different EM species (stars).

### 3.3.5 *Relationship of $^{15}N$ in mycorrhizal root tips and total fine root $^{15}N$*

In order to investigate whether high $^{15}N$ accumulation in mycorrhizal root tips resulted in an increased allocation of $^{15}N$ into fine roots, we determined the relationship between the $^{15}N$ signatures in both plant fractions.

We found a low positive relationship of $^{15}N$ signatures of EM root tips and mean fine root $^{15}N$ signatures, but no correlation between non-mycorrhizal root tips and fine root $^{15}N$ signatures (Fig. 3.5). These results indicate that mycorrhizal root tips might be more important for plant N uptake than non-mycorrhizal root tips.

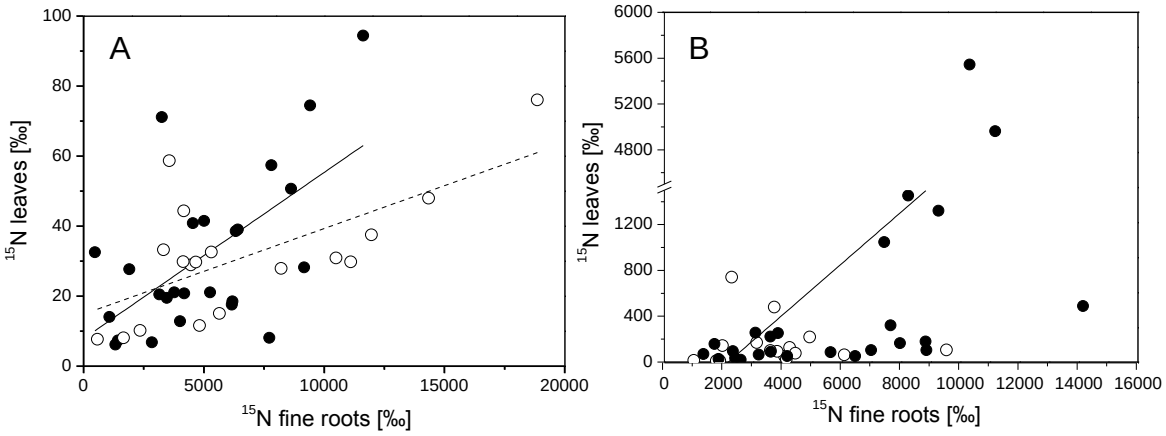



| Plant fraction | *y* | *P* | $R^2$ |
|---|---|---|---|
| EM root tip | *y*=4040+0.121*x* | 0.017 | 0.121 |
| NM root tip | *y*=-6010-0.001*x* | 0.988 | -0.048 |

**Figure 3.5:** Relationship between recently accumulated $^{15}N$ in fine roots and in EM fine root tips (filled dots, filled line) or NM root tips (open triangle).

### 3.3.6 *Relationship of $^{15}N$ signatures in fine roots and leaves*

Subsequently we investigated whether a higher fine root $^{15}N$ was related with an increased N accumulation in leaves. There was a positive relationship between $^{15}N$ signatures of fine roots and leaves, with an exception of ash in mixture (Fig. 3.6 A, B). This result suggests that a higher $^{15}N$ accumulation in fine roots might enhance the transport of N to leaves. In addition, partitioning of $^{15}N$ in tissues of ash changed in the presence of beech.




| Species | Treatment | *y* | *P* | $R^2$ |
|---|---|---|---|---|
| *F. sylcatica* | mono | $y$=7.8+0.005$x$ | 0.002 | 0.334 |
| | mix | $y$=14.9+0.003$x$ | 0.003 | 0.406 |
| *F. excelsior* | mono | $y$=-484.0+0.2$x$ | 0.008 | 0.222 |
| | mix | $y$=-2.1+0.1$x$ | 0.235 | 0.030 |

**Figure 3.6:** Relationship between recently accumulated $^{15}$N in fine roots and leaves of beech (A) and ash (B) grown in monoculture (filled dots, line) and mixture (open dots, dotted line).

### 3.3.7 *Short-term $^{15}$N and $^{33}$P supply in mycorrhizal and NM root tips*

Because EM species with high $^{33}$P accumulation were identified earlier (Chapter 2), we investigated whether high $^{33}$P accumulation in these EM species was related with a high nitrogen accumulation. Because no significant differences were found between EM species from monocultures and mixtures, the EM samples from beech monocultures and mixtures were pooled together. The EM species could roughly been divided to three groups (Fig. 3.7).

The first group contains EM species *T. castanea*, *T. badia* (n=2) and an unknown EM species MT8 (n=1), which had relative high accumulations of both recently acquired phosphorus ($^{33}$P) and $^{15}$N. The second group included species *P. involutus* and *Rhizoscyphus* sp., which do not accumulate significantly more $^{33}$P and $^{15}$N than vital or dry root tips. Thus, these species can be supposed to be relatively inactive for N and P uptake. The third group includes only one species, *Sebacina* sp. that had a relative high $^{15}$N accumulation, bur rather low $^{33}$P accumulation. These results indicate taxon related traits for N and P uptake within EM species.

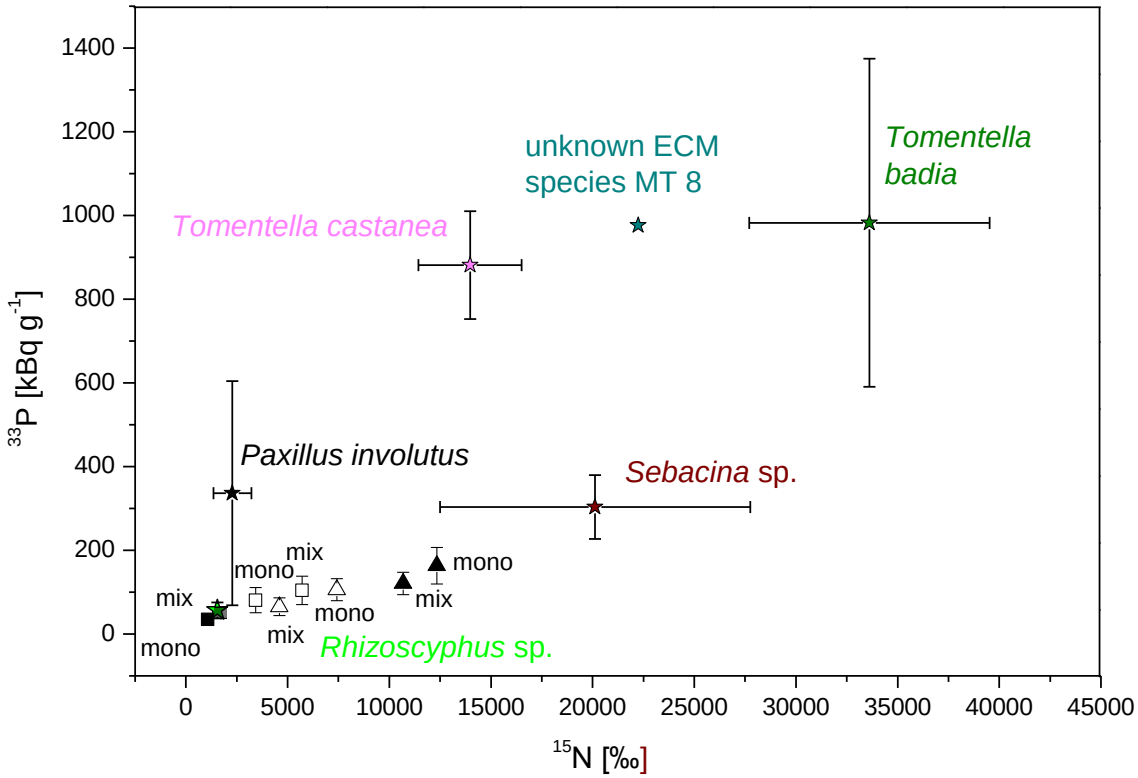



**Figure 3.7:** $^{33}$P concentration (kBq g$^{-1}$) and $^{15}$ N signatures (δ ‰) in ash (open symbols) and beech (filled symbols) root tips. Different symbols represent different fine root fractions: Dry root tips (squares), vital root tips of ash (open triangles), non-mycorrhizal root tips of beech (filled triangles) and beech root tips colonized with different EM species (stars). Root tips were divided in roots from monocultures (mono) and mixtures (mix), with an exception of EM root tips. Data indicateds means (± SE).

N and P accumulations of non mycorrhizal beech fine root tips and with vital, mycorrhizal ash fine root tips decreased in mixture. However, the difference between N/P ratios of monoculture and mixture were higher in non-mycorrhizal root tips of beech (difference, 12.7) than in root tips of ash (difference, 0.2). These results indicate that in mixture with ash, non-mycorrhizal beech root tips shift to P deficiency, whereas N and P supply of vital ash root tips was unaltered by the species identity of the neighbouring tree.

### 3.3.8 *Comparison of long-term and short-term N and P relations of fine roots and leaves*

The N and P accumulation in EM root tips might influence the relationship of N and P accumulation in other plant tissues. As an index of long-term N and P balance of the tree

species, N/P ratios of plant tissues were compared (Tab. 3.3). Subsequently the N/P ratios were compared with short-term $^{15}N$ and $^{33}P$ nutrition (Fig. 3.8 A, B).

N/P ratios of beech leaves were 16.1 in monoculture and 15.6 in mixture. These ratios suggest normal to optimal N/P balance of beech (Tab. 3.3). In contrast, leaf N/P ratios of ash were lower than 14 (mono: 11.5, mix: 12.4) indicated that ash was relatively more limited by N than by P.

**Table 3.3:** Balanced leaf N/P ratios of beech and ash based on leaf nitrogen and phosphorus threshold values suggested by Mellert & Göttlein (2012) and Kopinga & van den Burg (1995)

| Species | optimal leaf N mg $g^{-1}$ | optimal leaf P mg $g^{-1}$ | relative N deficiency | optimal N/P ratio | relative P deficiency | Reference |
|---|---|---|---|---|---|---|
| *F. sylvatica* | 19-23 | 1.2-1.9 | <10 | 10-19 | >19 | Mellert & Göttlein (2012) |
| *F. excelsior* | 23-28 | 1.4-1.6 | <14 | 14-20 | >20 | Kopinga & van den Burg (1995) |

Total N and P concentrations of ash leaves decreased in mixture (Fig. 3.8 A). In addition, the short-term N and P accumulation indicates that the nutrient allocation to leaves was affected by the presence of beech (Fig. 3.8 B).

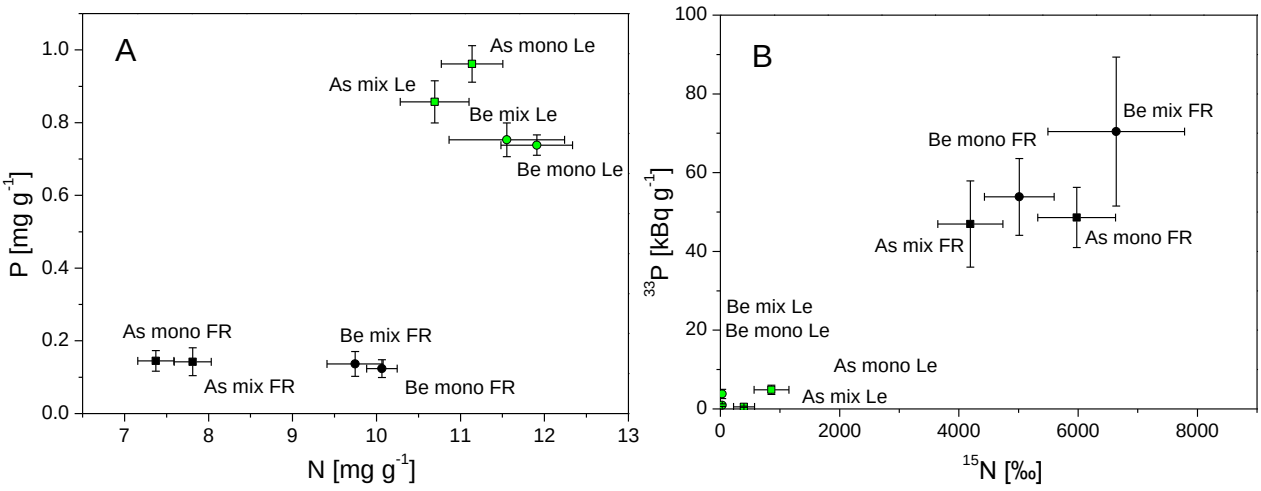



**Figure 3.8:** Long-term N and P supply (A), and short-term $^{15}N$ and $^{33}P$ supply (B) in leaves (Le) and fine roots (FR) of beech (Be) and ash (As) grown in monocultures (mono) and two-species mixtures (mix).

In fact, the difference value of leaf $^{15}N/^{33}P$ ratios in mixture and monoculture was 22 times higher in ash than in beech. Fine root $^{15}N$ and $^{33}P$ signatures of ash indicate that in the presence of beech N deficiency tended to increase more than P deficiency (Fig. 10B). In contrast, the growth in mixture with ash increased both $^{15}N$ and $^{33}P$ accumulation of beech fine roots.

## 3.4 Discussion

### 3.4.1 *Nitrogen uptake of beech and ash in heterospecific and conspecific pairs*

In accordance to our first hypothesis, the N deficiency of ash increased in the presence of beech. This was true although ash had a higher fine root biomass than beech. Experimental data and field surveys show that root biomass of ash tends to predominate in ash-beech mixtures (Jacob et al. 2012, Rust & Savill 2000). The results of the present study demonstrate that a higher root biomass of ash does not necessary result in a competitive advantage for N acquisition. In fact, beech was capable to compete with ash for N, presumably due to its EM association. N uptake of beech was, contrary to expected, unaffected by species identity of the neighbouring tree.

$^{15}N$ in fine roots and leaves tended to have a positive relationship. This result indicates that higher N uptake results in an increased leaf N accumulation. Only exception was ash in conspecific mixture. At eighth labelling day, leaf $^{15}N$ signatures of ash decreased in mixture. In contrast, $^{15}N$ accumulation increased in fine roots. These results might indicate that over the time N uptake of ash decreased in the presence of beech. Since the uptake kinetics was not measured in this study, this is only a speculation. The conclusions of van der Heijden et al. (200I) support our suggestion. Van der Heijden et al. (2001) demonstrated that in nutrient poor conditions, nutrient uptake of *Salix repens* increased faster in plants inoculated with AM than in plants with EM (van der Heijden et al. 2001). However, in long term plants benefitted more from EM association (van der Heijden et al. 2001). This long-term beneft of EM association may partially explain the well-known dominance of beech in mixed forests from the middle of the biostatic phase of forest development (Emborg 1998).

Total leaf N concentration of ash was higher than that of beech. Ash also accumulated more $^{15}N$ in leaves than beech. These differences occurred irrespective to the growth in mono-culture or in two species mixture, and can therefore be considered as taxon related characteristics. The higher N accumulation in leaves might result from the reported higher N uptake rate of ash compared with beech (Schulz et al. 2011). It has been suggested that high nutrient uptake kinetics might not be a competitive advantage in a nutrient depleted ecosystem (Aerts 1999). High nutrient content of leafs and high nutrient uptake rates are presumably

related to adaptation to nutrient rich ecosystems (Aerts 1999, Reich et al. 2003). Therefore, we suggest that ash is less adapted to nutrient depleted conditions than beech.

According to our knowledge, this was the first time that the N/P ratios of two competing tree species with different mycorrhizal types were investigated experimentally. N/P ratios reflect species specific differences and intraspecific responses to N and P supply (Güsewell 2004). Leaf N/P ratios demonstrate that contrary to ash, beech was able to maintain a balanced leaf N/P ratio in nutrient limited conditions. This might indicate that beech used N and P resources more economical than ash. N/P ratios (Fig. 11A) and relationship of short-term accumulation of $^{15}N$ and $^{33}P$ by ash suggest that ash was generally more limited by N than by P. However, both N and P deficiency of ash increased in mixture with beech. These results indicate that no clear niche differentiation between ash and beech occurred. Since tree interspecific interactions might shift with stand age (Cavard et al. 2011), adult trees in field could respond differently in comparable soil conditions.

### 3.4.2 *The role of EM species in plant N and P uptake*

EM association enhanced remarkably beech N and P supply. The comparison of N/P ratios of NM beech root tips in heterospecific and conspecific mixtures demonstrated that in the presence of ash non-mycorrhizal beech fine root tips shift to P deficiency. These results suggest that when the mycorrhizal colonization of beech decreases, it shifts to P deficiency. These results support the theory suggested by (Aerts 2002) that the interspecific competition with AM associated plant drives EM plant in non-mycorrhizal stage to P deficiency. In other words: the outcome of plant competition for N and P is driven by their mycorrhizal association.

The differences in N accumulation between EM species were species specific. EM with highest relative abundances; *Tomentella* spp., and *Sebacina* sp. had the highest $^{15}N$ signatures. Tomentella species are frequently found in EM root tips and in soil (Danielsen et al. 2012, Horton & Bruns 2001, Kjøller 2006, Pena et al. 2010). The reason for the dominance of Tomentella species is not known, but they are demonstrated as good competitors in root colonisation (Kennedy et al. 2007), which might have important ecological functions, such as efficient nutrient acquisition in N limited conditions (Horton & Bruns 2001, Lilleskov et al. 2002).

In contrast to the most abundant EM fungi in our experiment, *P. involutus* barely accumulated $^{15}N$. This was surprising, since it is well known species from N uptake experiments

(Arnebrant 1994, Kytöviita 2005, Kytöviita et al. 2001, Morel et al. 2006). We assume that the growth conditions might have affected nutrient uptake efficiency of *P. involutus*. This is supported by a survey of EM species abundance in forest sites with differing gradients of N deposition (Lilleskov et al. 2002). Dark mantled *Tomentella* species, similar to those abundant in our experiment, were suggested to be adapted to N uptake in N-poor soils; whereas *P. involutus* was suggested to be specialized to high N conditions (Lilleskov et al. 2002).

Consistent to our second hypothesis, species that were identified as high accumulative for P had often high accumulation of N (Tomentella spp. and unknown EM MT8). The second group of EM species (*Paxillus involutus* and *Rhizoscyphus* sp) had similar N and P ratios than non-mycorrhizal and dead fine root tips. These EM species did presumably not actively accumulate N or P. As a third variation, *Sebacina* sp. had a high N and an inter-mediate P accumulation. These differences in N and P acquisition suggest functional diversity of EM species. Nygren et al. (2008) and Nygren and Rosling (2009) showed that although many EM species share similar pools of genes for nitrate reductase (nar) and phosphomonoesterases, the growth of EM species differ significantly on supplied N and P sources. The authors infered continuously distributed traits to use different nutrient sources among EM species. Our results support this hypothesis.

## 3.5 Conclusions

We investigated N and P acquisition in beech and ash in nutrient limited conditions with emphasis on roles of different mycorrhizal species on plant N and P acquisition.

Both tree species related effects on N accumulation and indications of interspecific competition for N were found. The differences in total N contents of fine roots, as well as the transport of recently acquired N to leaves were tree species related.

The shift in N/P relation of ash fine roots and leaves indicated that N and P deficiency of ash increased in mixture with beech. Despite of long-term nutrient limitation, beech leaf N/P ratios remained balanced.

These results are particularly important, since they indicate that beech is better adapted to nutrient limited conditions than ash. We believe that this might be an important aspect to consider by establishing mixed forests stands with beech and ash saplings on nutrient limited sites.

The N and P relations differed greatly within EM species, indicating taxon related traits for N and P uptake. These results suggest that the species composition of root colonizing fungi is of great importance for nutrient acquisition of the host plant.

In conclusion, competition, rather than facilitation, for N and P occurred between beech and ash saplings in mixture. EM fungi contributed significantly to N and P acquisition of beech.

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# Chapter 4

# ECTOMYCORRHIZA AS A LINK BETWEEN TREES (*FAGUS SYLVATICA*) AND THE BELOWGROUND FOOD WEB

## 4.1 Introduction

Recently, there has been growing awareness of the relevance of ectomycorrhizal fungi (EM) to belowground food webs. Ectomycorrhiza play a key role in plant nutrient uptake and plant defence, but they also serve as an important channel of plant mediated carbon to soil food web (Pollierer et al. 2012). In temperate forests, ectomycorrhizal fungi contribute to 80% of the host plant nitrogen (van der Heijden et al. 2008). Up to 30% of the total photoassimilate carbon is transferred to the fungal symbiosis partner (Smith & Read 1997) and partitioned to three different pools: fungal biomass, exudation and respiration. Well reported is the high EM biomass in forest soils. The fungal mycelium comprises up to 70 mg $g^{-1}$ soil (Vinichuk & Johanson 2003) and Wallander et al. (2001, 2004) estimated total amount of EM mycelium including EM mantles up to 700–900 kg·$ha^{-1}$ (Wallander et al. 2004, Wallander et al. 2001), others suggest approximately 30% of the microbial biomass and 80% of the fungal biomass in boreal forest soils to be ectomycorrhizal mycelium (Högberg & Högberg 2002, Wallander et al. 2003, Wallander et al. 2001).

The metabolic activities among ectomycorrhizal tips differ largely. (Jany et al. 2003) measured with a microradiorespirometry assay respiration rates between 7 and 34 nmol $O_2$ $g^{-1}s^{-1}$ in ectomycorrhizal root tips of different EM species. High variability of respiration rates between single ectomycorrhizal root tips has also been found (Jany et al. 2003, Trocha et al. 2010).

Exudation is considered as an important component of EM's overall C budget (Fransson & Johansson 2010, Godbold et al. 2006, Högberg & Högberg 2002). Exudation serves the nutrient uptake by mineral weathering (Landeweert et al. 2001), mineralisation of organic polymers (Durall et al. 1994, Read & Perez-Moreno 2003), and is also required for the nutrient uptake process, metal detoxification and antimicrobial defence (Bais et al. 2006). The range of produced substances differs between EM species. Exudates consist mainly of low molecular weight organic acids, saccharides, amino acids and peptides but ectomycorrhizal root tips also release fatty acids, polymeric carbohydrates and different enzymes into the rhizosphere (Courty et al. 2005, Gadd 2007). Hence, EM hyphae presumably contribute in a considerable manner to the nutrition of soil animals and carbon cycling (Bonkowski et al. 2009, Landeweert et al. 2001, Langley et al. 2006).

Despite the increasing interest on connecting mycorrhizal symbiosis with belowground ecosystem functions, our knowledge about the relationship of EM and soil animals is limited.

Recent studies have shown that the number of soil animals depending on carbon from EM fungi has been underestimated (Pollierer et al. 2007, Pollierer et al. 2009). Spore findings of EM in guts of soil animals suggest that diverse species feed on EM and serve the spore dispersal of belowground fruiting EM (Lilleskov & Bruns 2005). Feeding experiments with axenic fungal cultures have shown soil fauna species to feed selectively on fungi (Hiol et al. 1994, Scheu & Simmerling 2004). However, due to the differences in EM metabolism in symbiotic stage and the large variety of EM species in natural communities (Lang et al. 2011) feeding choice experiments can hardly reflect natural behaviour of EM feeding soil animals.

$^{13}C$ and $^{15}N$ tracing is a useful method to investigate nutrient fluxes and trophic interactions. Feeding strategies and trophic relations of soil animals have been investigated by comparing both natural and experimentally enriched abundances of stable isotopes $^{13}C$ and $^{15}N$ in soil food web (Högberg et al. 2010, Pollierer et al. 2009, Scheu & Falca 2000, Wardle et al. 2011). Application of stable isotope tracers allows a time–integrated detection of trophic pathways for nutrients derived from specific sources. The majority of early fungal studies with stable isotopes was limited to measurements on aboveground fruit bodies (Gebauer & Dietrich 1993, Gebauer & Taylor 1999, Högberg et al. 1999). Meanwhile the focus of experiments is increasingly on belowground trophic interactions. Högberg et al. (2010) showed that $^{13}C$ levels of Collembola species increased within days after a $CO_2$ labelling (Högberg et al. 2010). Pollierer et al. (2007) identified different diets of soil arthropods such as leaf litter, root derived and soil carbon respectively. Though recent research using stable tracers has shown plant recent photoassimilates as one of the most important carbon source of soil animals, it remains unclear if mycorrhizal structures were the primarly diet. Numerous soil animals are considered as fungivorous (Pollierer et al. 2007), often without paying attention as to whether these fungi are mycorrhizal or saprotrophic. Moreover, the majority of experiments have been performed in arbuscular mycorrhiza comprising grassland ecosystems (Graham 2001, Hempel et al. 2009, Hoffmann et al. 2009, Koricheva et al. 2009). Because of the vast importance of EM for plant fitness, better understanding of the trophic interactions of soil animals and EM is of great relevance. Since previous research has pointed out the ability of mycorrhizal fungi to interact with and influence a number of predator species of plant feeding species (Bonkowski et al. 2009, Hempel et al. 2009, Hoffmann et al. 2011) the predator arthropods with a trophic link to ectomycorrhizas are of special interest.

The aim of this study was to determine carbon and nitrogen fluxes between beech trees (*Fagus sylvatica*), its mycorrhizal fungi and soil arthropods. We used $^{13}C$ enriched $CO_2$ and $^{15}N$ labeled $NO_3NH_4$ nutrient solution to label beech seedlings. Intact soil cores ensured vital beech plants and associated mycorrhiza as well as an undisturbed soil animal community. To analyse carbon and nitrogen allocation processes, we measured $^{13}C$ and $^{15}N$ label in leaves, stem, fine roots, mycorrhizal root tips, in addition to fine roots directly above the mycorrhizal root tip and in soil animals.

We hypothetized that (a) EM–colonized root tips are strong sinks for both C and N, (b) the accumulation of $^{13}C$ and $^{15}N$ in root EM tips is directly related to the exchange of C and N with the attached root and (c) ectomycorrhiza are an attractive nutrient source for a number of soil arthropod species.

## 4.2 Materials and Methods

### 4.2.1 *Plant material and experimental setup*

The sampling sites were deciduous forest stands in two parts (Thiemsburg and Lindig) of National Park Hainich, Thuringia, Germany (51°05′28″N, 10°31′24″E). The forest was unmanaged for at least four decades and is characterised by having a total annual precipitation of 600–670 mm and an annual mean temperature of 7.5–8.0 °C (Leuschner et al. 2009). Naturally regenerated seedlings of *Fagus sylvatica* with a height of approximately 40 cm were extracted within intact soil cores, transported to a greenhouse and placed in pots with diameter of 25 cm, a height of 45 cm and a drainage

A total of nine trees were exposed to $^{13}CO_2$ enriched air (1018 ± 340 ppm, $^{13}CO_2$, EURISO-TOP GmbH, Saarbrücken, Germany) for one growing season (24.04- 05.10.09) at an average temperature of 22.8 °C (± 2.8) and humidity of 71.8% (± 13). Contemporaneously, the plants were irrigated daily with a Hoagland–based nutrient solution containing 0.6 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.01 mM $FeCl_3$, 0.4 mM $K_3PO_4$, 1.8 µM $MnSO_4$, 0.064 µM CuCl, 0.15 µM $ZnCl_2$, 0.1 µM $MoO_3$, 0.01 mM $H_3BO_3$, 5 mM $NO_3NH_4$ and 0.1 mM $^{15}NO_3{}^{15}NH_4$ (Euriso–top, Saint–Aubin, Essonne, France). The soil moisture was kept at 30% during the experiment.

4.2.2 *Plant harvest and soil collection*

At the end of the experimental treatment (05.10.09), leaf and aboveground plant mass were weighted. The soil columns were divided into two depth fractions: 0–5 cm corresponding to the $A_1$ horizon, and 5– 21 cm corresponding to the top of the $A_2$ horizon. Soil fauna were trapped in the litter and upper soil layers. The roots in the lower soil layer (below 5 cm depth) were immediately washed with hand warm tap water, divided into coarse (> 2 mm) and fine root fractions and weighed. After removal of trapped animals the roots in the upper horizon were also washed and weighed. Both root fractions were stored at 4 °C in moist tissue paper in plastic bags for mycorrhizal analysis. Leaf morphology and area were analysed from flat–bed scanner images usingWinFolia 2005a,b (Régent Instruments Inc., Québec, QC, Canada). Subsequently, the plant material was dried for 4 days at 70 °C and the dry masses of all plant fractions were recorded. Soil and leaf litter were also collected, dried and used for further analyses.

4.2.3 *Soil animal harvest and identification*

Soil fauna were collected from the entire upper soil layer by using a heat gradient extractor (Kempson 1963), driven out into glycol and stored in 70% EtOH. Animals were counted and sorted to group level. Lumbricids, Araneidae, Isopoda, Chilopoda (*Strigamia accuminata*, Lithobiidae and Geophilidae), Diplopoda, Oribatida and Collembolans were determined to species level.

4.2.4 *Ectomycorrhizal identification and quantification*

Fine roots of nine beech trees were examined with a stereomicroscope (Leica M205 FA, Leica Microsystems, Wetzlar, Germany). To determine EM colonization of the root tips, five fine root fragments were randomly selected in each sample and 500 vital root tips were counted. The percentage of EM colonization was calculated as: EM root tips/(EM root tips + non mycorrhizal vital root tips)x100.

Mycorrhizal roots tips were classified using the previously described (Druebert et al. 2009, Lang et al. 2011). morphotyping system developed by Agerer (1987–2006). The abundance of each morphotype in each subsample was recorded, the morphotypes photographed (Leica DFC420 C, Leica Microsystems, Wetzlar, Germany) and collected for chemical and molecular analyses.

4.2.5 *Molecular identification of the EM species*

DNA was extracted from 10–40 mycorrhizal root tips per sample using innuPREP Plant DNA Kit (Analytik Jena AG, AJ Innuscreen GmbH, Jena, Germany) according to the manufacturer's instructions, with the exception that lysis buffer and protease were added to the sample before the homogenisation with a push rod. DNA was resuspended in 100 µl elution solution. A polymerase chain reaction (PCR) product of the complete region of the internal transcribed spacer (ITS) region was amplified with the primer pair ITS1f 5´–CTTGGTCATTTAGAGGAAGTAA–3´and ITS4 5´– TCCTCCGCTTATTGATATGC –3´ (White et al., 1990; Gardes and Bruns, 1993). The total volume of the PCR reaction was 25 µl, containing 10 x PCR buffer with $(NH_4)_2SO_4$ (Fermentas, St–Leon–Rot, Germany), 2 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM each primer and 0.5 U Taq–polymerase (Fermentas, St–Leon–Rot, Germany). The PCR was performed in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) with the following settings: initial denaturation at 95 °C for 60 s, denaturation at 94°C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 60 s. This was repeated for 35 cycles before the final elongation at 72 °C for 10 min. If the subsequent gel analysis revealed more than one PCR product the PCR products were cloned in *E. coli* TOP19 (Invitrogen, Carlsbad, California, USA).

The ligation and transformation of the ITS–PCR products was done with a pGEM®–T and pGEM®–T easy Vector system kit following the manufacter´s instructions (Promega Corporation, Madison, USA), with the exception that the litigation product was incubated 1 h at 16°C. For the transformation Luria Bertani–medium (10g/l tryptone, 5g/l yeast extract, 10g/l NaCl, 15g/l agar and 1l demineralised $H_2O$) with 100µg/ml Ampicillin, 200 mg/ml IPTG, and 20 mg/ml X–Gal in DMF was prepared. The PCR products were sequenced at the Department of Forest Genetics and Forest Tree Breeding, University of Göttingen with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA). The sequences were edited with Staden Package (4.10, http://staden.sourceforge.net) and multiple sequence alignments were made with Clustal W (http://align.genome.jp). For the fungal species identification, sequences were compared with NCBI Genbank (www.ncbi.nlm.nih.gov) and UNITE (Kõljalg et al. 2005) databases. If the score was higher than 700 bits and the homology more than 97% the species suggested in gene bank, preferably UNITE was used. If the score was 600–700, homology 95–97% and both databases suggested the same genus, the species was recorded as an unknown species of the suggested genus. The sequences with

lower scores or a database sequence identity limited to family level were recorded on the basis of the results of a phylogenetic analysis or remained as unknown mycorrhizal species.
A phylogenetic tree of the EM species based on an alignment of ITS 1 and 2 and 2.5 S sequences with a total alignment length of 659 bases was constructed using SeaView (4.2.12, http://pbil.univ-lyon1.fr/software/seaview.html) program (Gouy et al. 2010). A Maximum Likelihood phylogram using GTR model aLRT was created with the following settings: invariable sites were ignored, optimized across site rate variation with 4 rate categories, NNI tree searching operation, BioN starting tree option, optimized tree topology and 5 random sites. Bootstrap values were calculated with 100 replicates.

4.2.6 *Chemical analysis: $^{13}C$ and $^{15}N$ labeling*

Dry plant tissues (leaf, wood, coarse root, fine root) as well as aliquots of soil from upper soil layer and leaf litter were milled with a ball mill (Type MM 2, Retsch, Haan, Germany), dried for another 24 h at 70°C and kept in an exicator. Aliquots of the samples were weighed into tin capsules and the of $^{15}N/^{14}N$ and $^{13}C/^{12}C$ were analyzed with an isotope ratio mass spectrometer IRMS (Delta C, Finnigan MAT, Bremen, Germany) at the Centre for Stable Isotope Research and Analysis, University of Göttingen. All mycorrhizal species and 3–10 mm long fine root sections next to the mycorrhizal mantle, directly connected with a given EM were analyzed after weighting ca 1 mg directly into tin capsules.

The isotopic composition of plant tissues, mycorrhiza, soil animals, soil and above ground litter was calculated as

$$\delta_{sample\ i}[‰] = \frac{R_{sample\ i} - R_{standard}}{R_{standard}} * 1000$$

where $R_{sample\ i}$ and $R_{standard}$ represent the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios of the sample *i* and standards. Results are shown in the δ notation in ‰ relative to the international standard Vienna Pee Dee belemnite (V-PDB) and 28/29 ratio.

We used the isotopic label of the fine roots as a reference to remove the background variability of the $^{13}C$ label.

$$\Delta_{\ sample\ i}\ [‰] = \delta_{sample\ i} - \ \delta_{fine\ roots\ i}$$

Additionally the atom fraction expressed as percentages (atom %) was used to analyse the $^{15}N$ and $^{13}C$ levels of plant and mycorrhizal tissues.

### 4.2.7 *Statistical analysis*

The statistical computing was carried out with R software v.2.10.0 (The R Foundation for Statistical Computing, http://www.r–project.org). All data were tested for normal distribution using the Shapiro–Wilk test and for homogeneity of variances with the Levene test. To compare the carbon and nitrogen contents and enrichments in different plant fractions, mycorrhizal root tips, soil, litter and soil fauna, a linear model was used. Studentized residuals were detected with the Bonferroni Outlier test. Subsequently, a non–parametric Kruskal–Wallis test was used to test significant differences between means followed by multiple comparisons of the data with the package nparcomp based on Pearson's product–moment correlation and non–parametric Behrens–Fischer problem (Brunner and Munzel, 2000). Mean ± SE were calculated using original data. Spearman´s rank correlation was used to test the relation of $\delta^{13}C$ to $\delta^{15}N$ label in EM species and their corresponding fine roots, the relation of leaf area to $\delta^{13}C$ and $\delta^{15}N$ label in EM species. Regression analysis was used to test the relation of $\delta^{13}C$ and $\delta^{15}N$ label in mycorrhizal root tips (EM) and mycorrhizal root tip corresponding fine roots (RM). In all analyses, differences were considered significant when $P \leq 0.05$.

## 4.3 Results

### 4.3.1 *Carbon and nitrogen allocation between plant tissues and soil organisms*

17 different EM and 29 invertebrates were recorded (Supplement Table S1, S2). The vital root tips were on average 96% (± 3%) colonised with EM. 13 EM species were identified through ITS sequence data and phylogenetic analysis (Supplement Table S1, Supplement Fig. S2). Three of these were ascomycota (*Cenococcum geophilum*, *Peziza succosa* and the unknown ascomycet MT 17); the remaining species belonged to the basidiomyceta.

The $\delta^{15}N$ ratio was highest in EM colonized root tips and decreased along the uptake and transport route from roots to leaves (Fig. 4.1 A). The $\delta^{15}N$ label of the EM root tips ($P$= 0.003*)* and the fine roots associated with the mycorrhizal root tips were significantly higher ($P \leq 0.001$) than that of the other plant fractions (Fig. 4.1) or non-labelled beech fine roots in field conditions (7.21 ± 2 ‰) . The lowest $\delta^{15}N$ signatures were found in soil (252 ± 172 ‰) and leaf litter (607 ± 540 ‰), both enriched compared to field conditions, where litter layer $\delta^{15}N$ values of approximately –3.7 ‰ in beech dominated mixed forests have been recorded

(Scheu & Falca 2000). The $\delta^{15}N$ ratio of soil fauna was higher than that of soil (661 ± 553 ‰), but clearly below that of plant tissues and their associated EM (Fig. 4.1 A).

The stable isotope $\delta^{13}C$ ratio showed an increase from distant plant fractions such as the stem to the mycorrhizal root tip. Furthermore, the $\delta^{13}C$ label was significantly higher ($P \leq 0.001$) in the mycorrhizal root tips than in any other fraction (Fig 4.1 B). $\delta^{13}C$ label in soil fauna was higher than in soil or leaf litter (Fig 4.1 B), whose labels were low (–23 ± 3 ‰).

### 4.3.2 *EM mediated N uptake and transfer in relation to carbon incorporation*

A regression analysis showed a strong positive relationship between $\delta^{15}N$ label in the mycorrhizal root tips and mycorrhiza–attached fine roots ($y = 0.005 + 0.46x$, $r = 0.630$ $P = 0.0001$), whereas no relationship was detected between the $\delta^{13}C$ ratio in the mycorrhizal root tips and mycorrhiza attached fine roots ($y = 65.5+0.30x$, $r = 0.182$, $P = 0.088$).

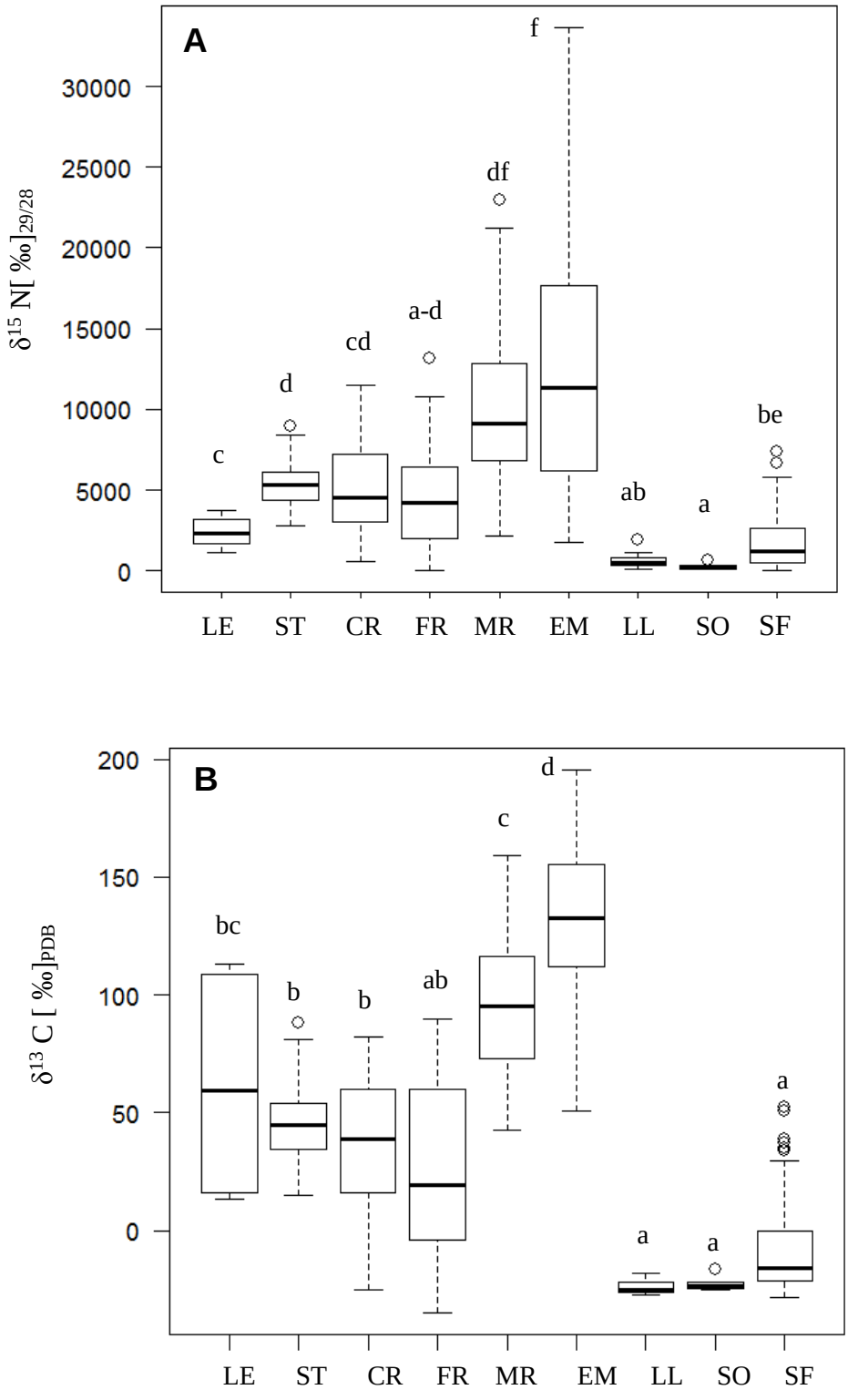


**Figure 4.1:** $\delta^{15}N$ (A) and $\delta^{13}C$ (B) in leaves (LE), stem (ST), coarse roots (CR), fine roots (FR), fine roots attached to mycorrhiza (MR), ectomycorrhizal root tips (EM), soil (SO), leaf litter (LL) and soil fauna (SF). The boxes indicate the 25th and 75th percentile, the horizontal line the 50th percentile and the whiskers within the 1.5 interquartile range.

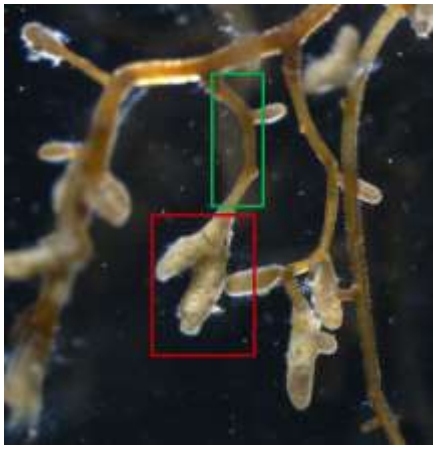

**Figure 4.2:** Mycorrhizal rot tip (red square) and fine root adjacent to mycorrhizal root tip (green square)

Since the acquisition of $^{15}N$ and $^{13}C$ may also be affected by the concentration of N and C in the tissues, the specific enrichment $^{15}N$ (atom%) and $^{13}C$ (atom%) was analysed (Fig. 4.2, 4.3). The $^{15}N$ enrichment in root adjacent to EM was strongly related to the $^{15}N$ label of the EM (Fig 4.3 A, $y = 1.44 + 0.54x$, $r = 0.750$, $P = 7.01 \times 10^{-6}$); whereas the $^{13}C$ enrichment in EM was not related to the $^{13}C$ enrichment in adjacent roots (Fig 4.3 B, $y = 0.84 + 0.30\ x$, $r = 0.184$, $P = 0.086$).

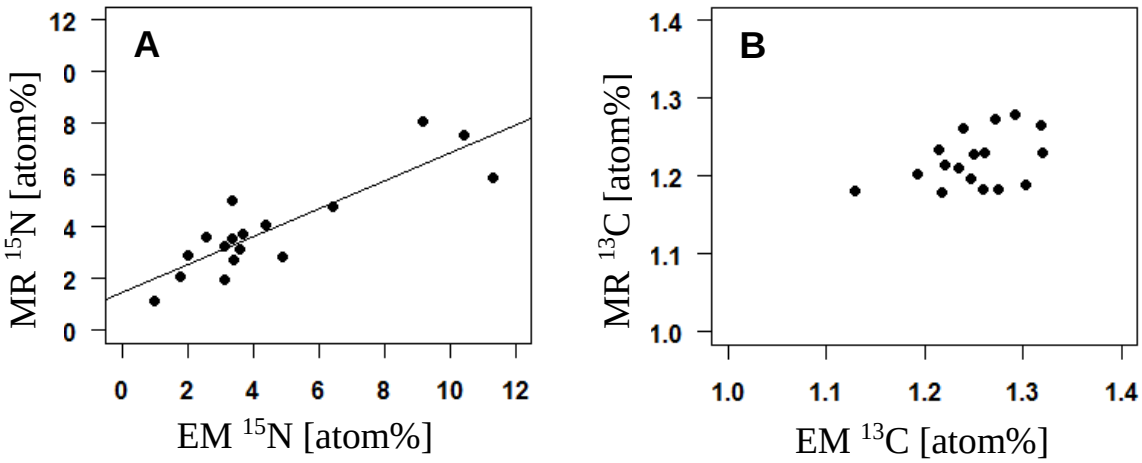


**Figure 4.3:** Relation between specific $^{15}N$ (A) and $^{13}C$ (B) signatures in ectomycorrhizal root tips (EM) and their corresponding fine roots (MR).

The $^{15}N$ enrichment in EM was not related to its $^{13}C$ enrichment ($y = 10201.23 + 19.53x$, $r = 0.006$, $P = 0.673$). Similarly, no relationship was found between the $^{15}N$ enrichment in mycorrhiza–attached roots and the $^{13}C$ enrichment in these roots ($y = 75.51 + 0.002x$, $r = 0.150$, $P = 0.092$).

We found a negative correlation ($P$ = 0.03, $r_s$=–0.46) between the $^{13}C$ enrichment in the mycorrhizal root tips and the specific leaf area (SLA). Also a correlation was found between $^{15}N$ enrichment of the mycorrhizal root tips and specific leaf area ($P$ =0.05, $r_s$= 0.42). However, this relation could not be followed up within one fungal species.

### 4.3.3 *Trophic relations of soil fauna, beech and ectomycorrhizal fungi*

EM root tips formed a group with the strongest increases in both $\delta^{15}N$ and $\delta^{13}C$ compared to roots and invertebrates (Fig. 4.4). The highest $\delta^{13}C$ label compared to fine roots was found in predators (–1.7 ± 29 ‰) followed by some species of decomposers soil animal taxa (–54.0 ± 36 ‰). Unexpected, putative fungivore species did have rather low $^{13}C$ label (–0.1 ± 29 ‰) (Fig. 4.4). Eight of the 29 invertebrate species displayed $\delta^{13}C$ enrichment compared to fine roots (Fig 4.4). These species included three species of the genus *Lithobius* (*L. erythrocephalus* 51.98 ‰, *L. curtipes* 27.22 ‰, and *L. forficatus* 19.51 ‰), which are predators. Other predators had significantly lower values (–31.98 ± 29.23 ‰). This result indicates that Lithobius species have a different diet than the other analysed predators. A wood louse *Trichoniscus pusillus* had a $\delta^{13}C$ level of 50.154 ‰ highly related to ectomycorrhizas.

Mesofauna species with the highest $\Delta^{13}C$ ratios were the oribatida mite *Hypochthonius luteus* (4.44 ‰) and two collembolan species (*Lepidocyrtus cyaneus* –17.52 ± 35.20 ‰ and *Sinella*/*Pseudosinella* –10.99 ‰). Endogeic earthworms (*Apporectodea rosea*, *Octolasion tyrtaeum*, 1.59 ± 0.18) had $\delta^{13}C$ ratios corresponding to fine roots. Also *Lumbricus rubellus* had a high $\Delta^{13}C$ ratio (19.0 ‰). The ratios of these three earthworms were higher than the values for the known anecic earthworm *Lumbricus terrestris* (–37.34 ± 26 ‰) and compost earthworms *E. octaedra* and *E. tetraedra* (–57.70 ± 17.02 ‰).

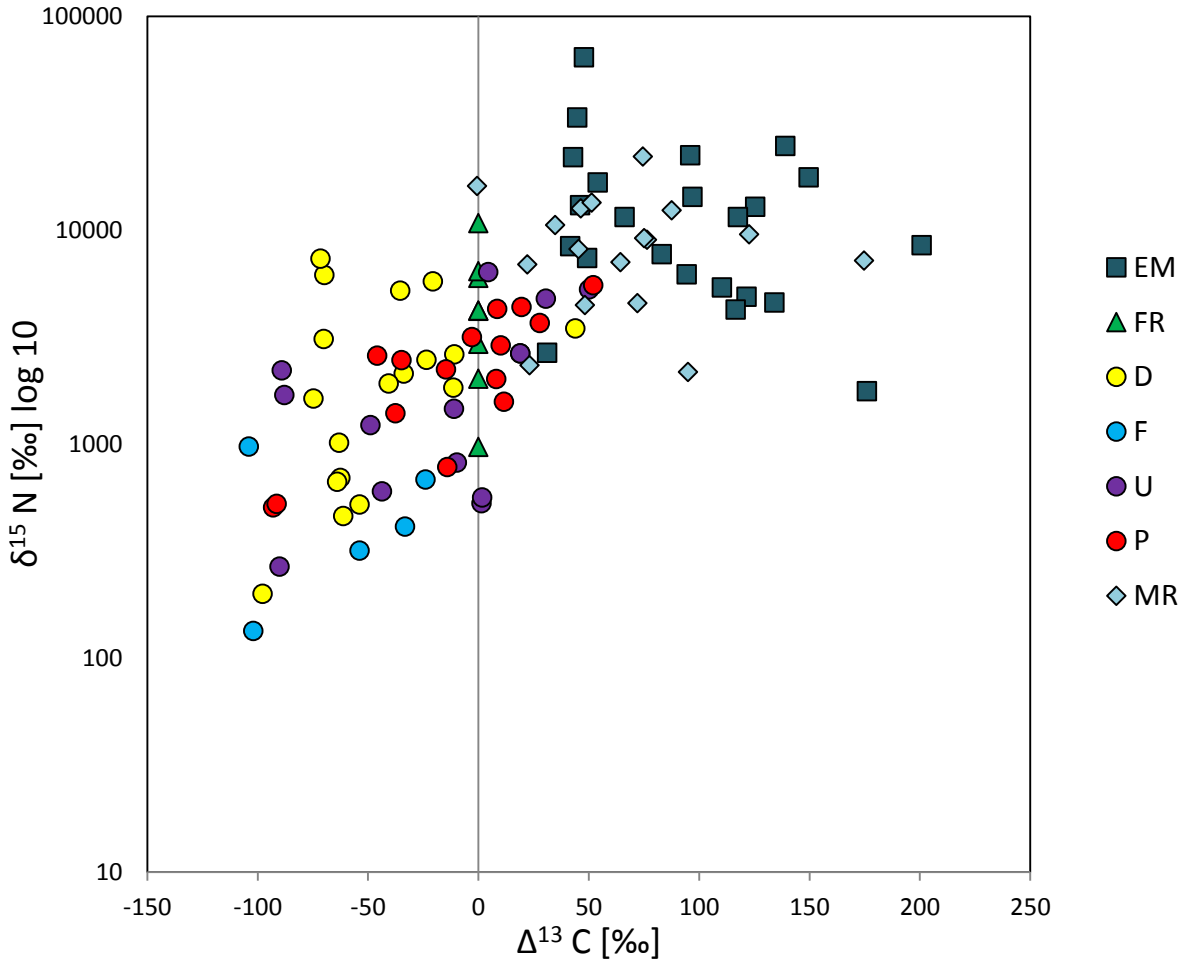


**Figure 4.4:** Enrichment of $\delta^{15}N$ and $\delta^{13}C$ through the food web. $\delta^{13}C$ signatures were normalized for fine roots.

Only one of the seven analysed oribatida mites, *H. luteus* had a $\delta^{13}C$ label higher than the fine roots (+ 4.44 ‰). The putative fungivorous oribatida species; *Nothrus palustris*, *Damaeidae* and myriapoda *Craspedosoma* sp. (–54.5 ± 33 ‰) did not show mycorrhiza–related $\delta^{13}C$ labels. Other oribatida mites of as yet unknown trophic level, i.e., *Hermaniella* sp. and *Xenillus* sp. had ratios near to known detritus–feeding oribatida (–69.9 ± 0.1 ‰). This would indicate that these species are not exclusive mycorrhiza feeders, but rather detritivores.

## 4.4 Discussion

### 4.4.1 *The experimental setup maintain a fungal beech EM community*

Experiments based on interactions between mycorrhiza and root–feeding soil fauna are often done in laboratory conditions with sterilised soil and controlled inoculation of fungi and insects, or in the field using fungicides. Both methods can have unwanted effects on the system leading to bias in the experimental setup (Koricheva et al. 2009). Furthermore pronounced host–fungal preferences exist (Lang et al. 2011), and the effects of the symbiosis can differ between fungal strains (Nygren & Rosling 2009). In spite of the limitations of *in vitro* experiments, Johnson et al. 2012 emphasized the need of approaches under controlled conditions for resolving ecological questions.

In order to maintain a natural rhizosphere we used naturally regenerated seedlings from a forest that were kept in intact soil cores with their natural soil community. However, elevated $CO_2$ used could have lead to shifts in the carbon allocation process, mycorrhizal biomass and community structure (Fransson 2012, Parrent & Vilgalys 2009, Wiemken et al. 2001). Regardless of this, five months after the initiation of our greenhouse experiment mycorrhizal colonisation of the plants was comparable to that found in natural forests (Lang et al. 2011). *C. geophilum, L. subdulcis* and *Tomentella* spp. were the most frequently recorded species on beech roots. These and other species found have been reported as EM of beech trees in Hainich national park (Lang & Polle 2011) and therefore we assume that the EM community was similar to the natural stand of the beech trees.

### 4.4.2 *High nitrogen accumulation in ectomycorrhizal fungi*

We found an increasing shift of $\delta^{13}C$ and $\delta^{15}N$ from plants to fine roots attached to mycorrhiza. This demonstrates that a considerable part of the nitrogen taken up by the EM is directly transported to the plant and not fixed in the hyphal structures. On the other hand, EM is a strong sink of plant–mediated carbon. Similar to Högberg et al. (2008), we found highly heterogeneous $^{13}C$ levels in EM root tips. The carbon productivity of plants has been shown to depend, amongst other factors, on specific leaf area (McMurtrie & Dewar 2011). In our experiment $^{13}C$ enrichment in the mycorrhizal root tips was negatively related to specific leaf area. At high nutrient availability plant growth is rather carbon–limited; therefore it has been

suggested, that bigger plants might have less carbon available for investment to mycorrhizas (Yanai et al. 1995). In contrast, the $^{15}N$ labels of the mycorrhizal root tips were positively correlated to SLA.

No correlation between $\delta^{15}N$ and $\delta^{13}C$ enrichment of the EM was found. This result is in disagreement with Högberg et al. (2008), who found a positive correlation of N and C labels in ECM root tip. A longer labelling period and a higher label of our experiment compared to the experiment of Högberg et al. (2008) might influence the relation of nitrogen and carbon in mycorrhizal root tips differently. Furthermore, our results indicate that the plant leaf area or other factors affect plant carbon allocation and nitrogen uptake. For instance the patchiness of the available nitrogen in the soil can contribute to the differences in the nitrogen concentration of the mycorrhizal root tips and in carbon allocation processes (Corrêa et al. 2011, Kytöviita 2005, Rosling, Lindahl & Finlay, Wallander et al. 2002). In our study, however, the plants were regularly irrigated with $^{15}N$ fertilizer, and we therefore assume no high patchiness of the $^{15}N$ label.

The $\delta^{13}C$ enrichment differed between EM species up to 50 %. Previously we demonstrated that plant productivity and the amount of plant mediated carbon affect the carbon allocation to the mycorrhizal root tips (Druebert et al. 2009). Ecological theories hold that carbon investment is higher to more beneficial fungal associates (Bruns et al. 2002, Corrêa et al. 2008), however, studies with contrasting results exist. Plant belowground carbon allocation was showed rather to relate with soil nitrogen availability than with actual nitrogen gain through mycorrhiza. Also indications that host plants continue to invest carbon to mycorrhiza by decreasing nitrogen supply has been found (Corrêa et al. 2011, Corrêa et al. 2008). The individual root tips have been suggested to receive different amounts of carbon depending on mycorrhizal species or intra specific differences between fungi (Johnson et al. 2012). In our experiment, low numbers of individual species precluded statistical analysis of this phenomenon.

Nevertheless, we presume that EM species differ in their ability to allocate nitrogen to the host. If plants provide mycorrhiza with more efficient nitrogen supply with increased carbon allocation we would expect a direct correlation of carbon and nitrogen labels found in mycorrhizal root tips. In our experiment however nitrogen and carbon allocation were not directly related. This leads to the conclusion that plant carbon allocation did not depend on nitrogen label of mycorrhizal root tips or *vice versa*. However, the fact that nitrogen and

carbon allocation were not directly related could indicate that EM with lower nitrogen accumulation allocate other resources such as other nutrient elements or water (Rosling, Lindahl & Finlaya, Rosling, Lindahl, Taylor b, Wallander et al. 2002).

4.4.3 *EMs have a high nutritional value*

We showed that the mycorrhizal root tips and the fine roots attached to the mycorrhiza had the highest C and N enrichment of the entire root system. This would make root tips nutritionally more attractive to root feeders. In fact, herbivore soil animals have been reported to prefer to feed on root tips (Bonkowski & Roy 2005, Rodger et al. 2003).

We found soil fauna species with an enriched $\delta^{13}C$ signature compared to fine roots, but the enrichment was on average five times lower than the label found in mycorrhizal root tips (96.86 ± 38.69 ‰ vs. -26.52 ± 38.41 ‰). The highest $\delta^{13}C$ label compared to fine roots was found in predators followed by fungivore species. The decomposers had the lowest $\Delta^{13}C$ values. The species with currently unknown trophic position were positioned between decomposers and putative fungal feeder, suggesting these species to have a mixed diet.

In this experiment we measured the EM $^{13}C$ and $^{15}N$ labels in mycorrhizal root tips. Thus a distinction between labels in different mycorrhizal structures was not possible, the separation of mycorrhizal root tips and extramatrical mycelium as food source of soil animals was partly based on available information about the behavior and ecology of the found soil animals.

The most similar isotopic $\delta^{13}C$ ratios to mycorrhizal root tips were found in macrofaunal species, which have no direct trophic links to mycorrhizas. The isotopic $\Delta^{13}C$ ratios of the predatory species *Lithobius erythrocephalus* (51.98 ‰), *Hypochthonius luteus* and an isopoda species, *Trichoniscus pusillus* (50.15 ‰) corresponded with the $\Delta^{13}C$ label of ectomycorrhizal root tips. *T. pusillus* have been earlier shown to feed on litter colonizing microbiota (Kautz et al. 2000). Presumably leaf litter grazed by *T. pusillus* could be incorporated with ectomycorrhizal mycelia (Hrynkiewicz et al. 2010, Perez-Moreno & Read 2000), but also other carbon sources like the bacterial food channel cannot be excluded.
*L. erythrocephalus* and *H. luteus* have indirect trophic links to mycorrhizas through feeding on mycorrhizal–nourished prey (Maraun 2012). Isotopic values of predators and secondary decomposers have been found to resemble each other (Maraun 2012). We found a significant difference between $\Delta^{13}C$ values of these trophic groups. Predators with intermediate $^{13}C$ enrichment presumably feed on mixed prey. Our results suggest EM to be a possible carbon

source for both trophic groups. Still, explicit work is needed to distinguish the bacterial and fungal nutrient channels.

The comparison of $\Delta^{13}C$ ratios of anecic earthworm *L. terrestris* (–37.34 ‰) with earth worms from other ecotypes (compost earthworms, epigeic earthworms and endogeic earthworms) showed that the endogeic species *Apporectodea rosea* and *Octolasion tyrtaeum* had a $\delta^{13}C$ enrichment similar to that of fine roots. However these results are based on single measurements. In contrast, the epigeic Eiseniella species *E. octaedra* and *E. tetraedra* (–57.70 ± 17.02 ‰) have much lower $\Delta^{13}C$ values. This is in accordance with an earlier suggestion that these species feed presumably on leaf litter (Scheu & Falca 2000). Another epigeic species *Lumbricus rubellus* differed clearly from these two species, by having a higher $\Delta^{13}C$ ratio (19.0 ‰). *A. rosea* and *O. tyrtaeum* have been suggested to feed on older and more humified litter (Maraun 2012). Our findings suggest endogeic and epi–endogeic earth worms to have a possible indirect link to plant carbon, which might rely on older litter with incorporated EM mycelium or recalcitrant carbon from dead hyphae in deeper soil layers (Langley et al. 2006).

An oribatid mite from the Family Hypochthoniidae, *Hypochthonius luteus* (4.44 ‰), showed a fine root related enrichment of the $\delta^{13}C$ label. We expected putative fungivore oribatid mites *Nothrus palustris* (–53.74 ± 35.75 ‰) and *Damaeidae* (–77.95 ± 24.06) to be enriched in $\delta^{13}C$ compared to fine roots. However, no corresponded $\delta^{13}C$ enrichment was found. We suggest both species to feed on saprophytic fungi or litter.

We found two collembolan species from the family Entomobryoidea, *Lepidocyrtus cyaneus* (–17.51 ± 35.20 ‰) and *Sinella*/*Pseudosinella* spec. (–10.99 ‰), enriched with $\delta^{13}C$. Högberg et al. (2010), found that Collembolan species from the family Entomobryoidea are rapidly labeled with tree derived $^{13}C$, and suggested that they feed upon highly active mycelia. Because the label levels were much higher than in the leaf litter (Fig. 4.4), but lower than in EM, we presume that Entomobryoidea might have a mixed diet that includes mycorrhizas, fine roots or both. This result is in accordance with Pollierer et al. (2007) who suggested Entomobryidae (Collembola) to acquire root and leaf litter derived carbon in similar amounts.

## 4.5 Conclusions

Our results show that the mycorrhizal root tips and the fine roots attached to the mycorrhizas have the highest $^{13}C$ and $^{15}N$ enrichment compared to other plant structures. Also we found a strong relationship between nitrogen concentration in mycorrhizal root tips and adjacent fine roots. This indicates that a notable amount of nitrogen in EM is directly allocated to the host plant. Similar relations were neither found between carbon concentrations in mycorrhizal root tips and adjacent fine roots, nor between carbon and nitrogen enrichment in mycorrhizal root tips. Due to the high nitrogen concentrations in mycorrhizal root tips EM can be concidered to have a high nutritional value. Our results demonstrate that soil animal species from all trophic levels potentially connect to EM fungi. However, explicit work is still needed to distinguish between bacterial and fungal nutrient channels, as well as between mycorrhizal root tip and EM mycelia as nutrient sources of soil animals. Finally, EM fungi are pivotal as a nutrient channel not only to the host plant but also for the soil food web.

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Supplementary data

Supplement Table S1: Molecular information of ectomycorrhizal species. Species were identified by ITS sequencing and sequence information was deposited in NCBI databank. If the homology was higher than 94% and the score higher than 700 bits, the name suggested by the database, preferentially that of UNITE was accepted. If both NCBI and UNITE database did suggest the same genus, species with higher score than 600 bits were named as unknown species of the suggested genera, unknown species clustering with known species were named as unknown species of the same genera, otherwise unknown ectomycorrhizas were called unknown species with an internal morphotype number (MT). Species for which sequence information was not available were called by their internal morphotype number (MT). ACC = Accession number in NCBI databank, Best BLAST match = name obtained from NCBI or UNITE, UncECM= Uncultured Ectomycorrhiza.

Supplement Table S2: Taxonomical (order/phylum) and ecological (ecology/diet) information of soil arthropods and their $\Delta^{13}C$ enrichment. $\delta^{13}C$ signatures were normalized for fine roots.

Supplement Figure S1: Phylogeny of EMF species based on ITS sequences. Maximum Likelihood , ML ln(L)= 4066.9, 657 sites GTR 4 rate classes. The Bootstrap values were generated from 100 replicates

**Supplement Table S1:**

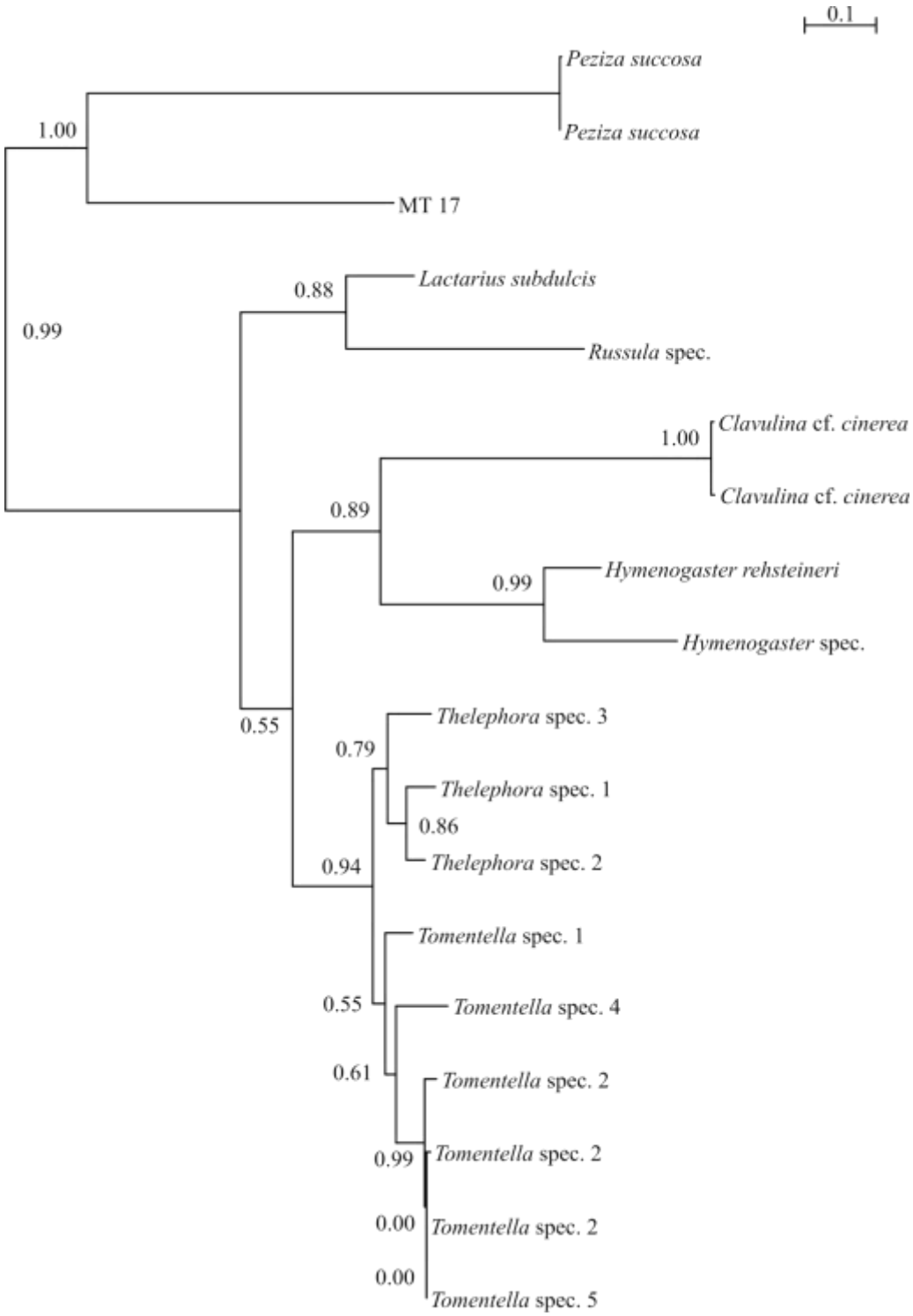
| Species | NCBI Genbank ACC number | Best BLAST Match | Source | Source Accession Database number Best BLAST Match | Length of Fragment | Homology [%] | Score [bits] |
|---|---|---|---|---|---|---|---|
| *Tomentella* spec. 1 | JQ982963 | Uncultured Thelephoraceae clone SC_ITS_157 | NCBI | GQ219960.1 | 685 | 100% | 1266 |
| *Tomentella* spec. 2 | JQ982967 | Uncultured ectomycorrhiza (Thelephoraceae) clone M44C6 | NCBI | FJ196997.1 | 629 | 98 | 830 |
| *Tomentella* spec. 4 | JQ982968 | Tomentella galzinii | UNITE | UDB000263 | 553 | 95 | 844 |
| *Tomentella* spec. 5 | JQ982969 | Uncultured Thelephoraceae clone 6S4.22.S04 | NCBI | EF619818.1 | 256 | 93 | 856 |
| *Thelephora* spec.1 | JQ982964 | Uncultured ectomycorrhiza (Thelephoraceae) clone OT-76 | NCBI | FJ0130.1 | 475 | 100 | 878 |
| *Thelephora* spec. 2. | JQ982978 | Uncultured Thelephoraceae clone 6S4.22.S04 | NCBI | GQ219960.1 | 568 | 99 | 1031 |
| *Clavulina* cf. *cinerea* | JQ982970 | Clavulina cf. cinerea BIO 10304 | NCBI | EU862226 | 564 | 99 | 876 |
| *Hymenogaster* spec. | JQ982975 | Hymenogaster vulgaris voucher RBG Kew K(M)27363 | NCBI | EU784365.1 | 472 | 95 | 725 |
| *Hymenogaster rehsteineri* | JQ982973 | Hymenogaster rehsteineri isolate zb20070814 | NCBI | GU479324.1 | 572 | 100 | 1057 |
| *Lactarius subdulcis* | JQ982974 | Lactarius subdulcis | UNITE | UDB000380 | 394 | 100 | 781 |
| *Russula* spec. | JQ982979 | Russula romellii 2-1119IS77 | NCBI | AY061714.1 | 388 | 97 | 652 |
| *Pestiza succosa* | JQ982977 | Peziza succosa voucher KH-98-07 (C) | NCBI | DQ200840 | 586 | 99 | 1075 |

**Supplement Table S2:**

| Phylum/ Order | Species | $\Delta^{13}C$ [ ‰] PDB | | Diet | Trophic level |
|---|---|---|---|---|---|
| Arachneida | *Clubiona* sp. | -14.66 | | mixed prey* | predator |
| Arachneida | *Hahnia pusilla* | -20.22 | ± 17.4 | mixed prey* | predator |
| Coleoptera | *Coleoptera A* sp. | -29.54 | ± 44.8 | mixed diet* | unknown |
| Collembola | *Lepidocyrtus cyaneus* | -17.52 | ± 35.2 | detridivore | secondary decompose |
| Collembola | *Sinella/Pseudosinella* ssp. | -10.99 | | mixed diet* | secondary decomposer |
| Isopoda | *Trichoniscus pusillus* | 50.15 | | litter with incorp EMF mycelium* | secondary decomposer |
| Lumbricida | *Aporrectodea caliginosa* | -66.10 | ± 22.5 | leaf litter* | primary decomposer |
| Lumbricida | *Apporectodea rosea* | 1.41 | | leaf litter/fine root litter* | secondary decomposer |
| Lumbricida | *Eiseniella octaedra* | -40.68 | | leaf litter* | primary decomposer |
| Lumbricida | *Eiseniella tetraedra* | -74.73 | | leaf litter* | primary decomposer |
| Lumbricida | *Lumbricus terrestris* | -37.34 | ± 25.9 | leaf litter | secondary decomposer |
| Lumbricida | *Lumbricus rubellus* | 18.96 | | old leaf litter with incorp EMF mycelium* | secondary decomposer |
| Lumbricida | *Octolasion tyrtaeum* | 1.77 | | old leaf litter with incorp EMF mycelium* | primary decomposer |
| Myriapoda | *Craspedosoma sp.* | -34.17 | | leaf litter /saproph. fungi* | secondary decomposer |
| Myriapoda | *Glomeris helvetica* | -93.00 | | leaf litter* | primary decomposer |
| Myriapoda | *Glomeris undulata* | -41.70 | ± 49.7 | leaf litter* | primary decomposer |
| Myriapoda | *Lithobius curtipes* | 27.77 | | fungivore prey* | predator |
| Myriapoda | *Lithobius erythrocephalus* | 51.98 | | fungivore prey* | predator |
| Myriapoda | *Lithobius forficatus* | 19.51 | | fungivore prey* | predator |
| Myriapoda | *Lithobius muticus* | -12.32 | ± 22.5 | mixed prey* | predator |
| Myriapoda | *Necrophleophagus* | -9.97 | ± 23.0 | mixed prey* | predator |
| Oribatida | *Achipteria* sp. | -70.92 | ± 19.2 | leaf litter | primary decomposer |
| Oribatida | *Damaeidae* sp. | -77.95 | ± 24.1 | leaf litter* | primary decomposer |
| Oribatida | *Hermaniella* sp. | -69.77 | | leaf litter* | primary decomposer |
| Oribatida | *Hypochthonius luteus* | 4.44 | | fine roots* | primary consumer*predator |
| Oribatida | *Nothrus palustris* | -53.74 | ± 35.8 | saphrohytic fungi* | Primary decomposer |
| Oribatida | *Platynothrus peltifer* | -71.60 | | decomposer | primary decomposer |
| Oribatida | *Stegacarus magnus* | -27.99 | ± 7.4 | leaf litter | primary decomposer |
| Oribatida | *Xenillus* sp. | -70.05 | | detritus | decomposer/detritivore |

$\Delta^{13}C$ values normalized for fine roots. Data shows means (± SD). Data without SD is based on a single measurement. All diets after corresponding references, with the exception of species with to our knowledge at the time unassured diet. Ecology/ trophic level marked with an asterix indicate diet/ecology suggested by author.

**Supplement Figure S1:**



# 5. General discussion

## 5.1 *Differential interactions between mycorrhizal fungi and tree species impact the structure and dynamics of plant communities*

The present work demonstrates that interactions between ash and beech, in respect to nitrogen and phosphorus acquisition, were notably supported by mycorrhizal fungi. This resulted in a disadvantage for ash.

In mixed ash-beech forests, ash tends to dominate the belowground area with higher root biomass (Jacob et al. 2012, Rust & Savill 2000). This thesis showed that despite the generally higher fine root biomass of ash, its N and P limitation increased in the presence of beech (Chapter 2 and 3). This was surprising, because ash has been reported to take up N (and P) more effectively than beech (Schulz et al. 2011). There are two possible explanations for the decreased growth and nutrient accumulation of ash in mixture with beech. Firstly, the results indicate that the interaction with beech affected nutrient accumulation of ash. The comparison of nutrient uptake efficiencies between tree species is usually based on measurements in monocultures (Comas et al. 2002, Schulz et al. 2011). However the conclusions based on plant functions measured in monocultures may not display the plant reactions in species mixtures.

Secondly, efficient nutrient retention and economical resource use might be more important plant characteristics in nutrient limited conditions than high nutrient uptake kinetics (Aerts 1999). The maintained N/P balance of beech leaves compared with an N/P inbalance of ash in the species mixture might therefore indicate that beech is better adapted to nutrient limitation than ash.

Our findings are important, since they suggest that despite the often higher root mass, ash cannot be interpreted as a belowground dominating tree species in mixture with beech. The relative competition index for P presented a convincing argument for the contrary.

The increased root biomass of beech in a dual-splitroot-rhizothron experiment indicates belowground overyielding, and thus, a possible biodiversity effect (Appendix 1). The previous studies of belowground diversity of tree species report both, positive (Schmid & Kazda 2002) and neutral (Bolte & Villanueva 2006, Leuschner et al. 2001, Meinen et al.

2009) biodiversity effects on root growth. However, the role of tree species identity rather than biodiversity might predominate the effects (Jacob et al. 2012).

The differences in fungal species composition (Chapters 2, 3 and 5) might also change the strength of the plant interactions. This would also explain the different outcomes of studies on soil N form preferences for ash and beech (Dannenmann et al. 2009, Gessler et al. 1998, Schulz et al. 2011, Stadler et al. 1993).

No comparable biomass increase of beech, as found in dual-splitroot-rhizothron experiment, occurred in more nutrient limited system (Chapter 2 and 3). This result suggests that overyielding may be possible when resource limitation does not drive the tree species to competition. In fact, model based analysis of tree species diversity on temperate forest productivity indicate that environmental conditions influence productivity of mixed tree sites (Morin et al. 2011).

In addition, the differences in growth responses in our experiments could be explained by limited rooting area. In order to drive competition, the available soil volume was clearly restricted in chapters 2 and 3. Here, fine root density was on its upper limit in both monocultures and mixtures after two growth periods (1 g FR dw $l^{-1}$soil $pot^{-1}$, $P$ = 0.511). Similar upper fine root densities (1-2 g biomass $l^{-1}$) have been reported in the topsoil horizons of temperate-broad leaved forests (Hertel 2011). This density appears to be exceeded neither in monocultures nor in mixed stands (Jacob et al. 2012). In contrast, in the split-root experiment the soil space compared to root mass was larger. This might partially explain the facilitative effect of ash on growth of beech, whereas growth of ash was unaffected by the presence of beech.

This thesis indicates that EM fungi contributed significantly to both N and P acquisition of beech. In addition, we demonstrated that EMs were substantially better accumulators for both, N and P, than ash root tips (Chapter 2 and 3). Our findings reinforce earlier studies on dual-mycorrhizal associations of single tree species, which have suggested that EM fungi are more efficient providers of P than AM fungi (Jones et al. 1998, van der Heijden 2001), but contrast the widely held view that AM fungi are more important for plant P nutrition than EM.

Although the biomass of extraradical hyphae of AM and EM fungi were not measured in this study, it is likely that EM rhizomorphs were more present in soil than extraradical hyphae of AM fungi (Chapter 2 and 3). This suggestion is supported by estimations of abundances of

fungal species with different ecological lifestyles in soil (Bueé et al. 2009, Danielsen et al. 2012). Similar accumulation of P in EM rhizomorphs and in EM root tips suggests that extraradical hyphae of EM were highly involved in beech nutrient uptake.

The distinction of extraradical hyphae of AM and EM species and their quantification are rather challenging. The currently available methods such as phospholipid fatty acid markers (PLFAs), content of ergosterol and quantification of ITS sequence copy numbers have only limited usability to differ between EM and AM types. We can therefore only speculate that a higher extent of extraradical EM hyphae may have lead to retention of nutrients in EM structures.

The differences in nutrient accumulation of EM species were taxon related (Chapters 2, 3 and 5). The most abundant beech root colonizing EM fungi, *Tomentella castanea* and *Sebacina* sp., were concurrently high accumulative species for both, N and P (Chapter 2 and 3). In contrast, N and P accumulation of *Paxillus involutus* was comparable with non-mycorrhizal root tips. This was surprising because this species has been reported to take up and translocate N to *Fagus sylvatica* (Finlay et al. 1989) and P to *Pinus sylvestris* (Bücking & Heyser 2000). In previous studies, narrow niche-differentiation within lineages of EM species has been demonstrated (Geml et al. 2008). Therefore, relatively low N and P accumulation in the present experiments might indicate an unprofitable host-fungus association. Another explanation could be that the growth conditions were not suitable for *P. involutus*. Since host plants transfer more C to those fungal species that provide more nutrients (Corrêa et al. 2011, Corrêa & Martins-Loução 2011, Kiers et al. 2011), unequal partitioning of carbon may have decreased the colonization of roots by less profitable EM species such as *P. involutus*.

Our data support that in a mixed ash-beech forest stand beech associated with EM community rather than ash with AM community has a competitive advantage in P and N acquisition. Highly nutrient accumulative EM species colonized up to 80% of mycorrhizal root tips of beech and were significantly involved in beech N and P acquisition. Given the high diversity in natural EM communities, complementarity of fungal species with respect to functional ecophysiological traits may promote coexistence of tree species (van der Heijden et al. 2003).

The results of the present study contributed new information about the role of different mycorrhizal fungi on plant competition for nutrients that so far has been lacking. In addition, the results of plant interactions between ash and beech on different soil nutrient availabilities could be applied to spatially explicit modelling of neighbor effects on forest dynamics.

## 5.2 *Outlook*

Intepretations of the functional significance of mycorrhizal fungi on plant interactions are still limited by a lack of knowledge about functional capacities of most mycorrhizal fungi. In the present work, functional relevance of EM species on plant N and P uptake was demonstrated. Amongst others, a high accumulation of $^{33}P$ in EM rhizomorphs was shown. More definite conclusions about the role of AM and EM fungi on nutrient acquisition of ash and beech will be possible when future work includes quantitative measurements of extraradical mycelium in soil. This may explain whether EM fungi use the given soil volume more efficiently than AM fungi and restrict the amount of available nutrients for ash. Also the quantification of mycelial biomass of AM and EM could be conducted with real time PCR using AM and EM specific primers. In the near future, a large set of mycorrhizal genomes will be available for future research. At the moment whole genomes of *Laccaria bicolour* and *Tuber melanosporum* (Martin et al. 2008, Martin et al. 2010) already enable a primer design for regions coding for N and P transporter. A use of degenerative primers could allow a screening of a large set of root colonizing fungi for the presence of these genes. These could be used to detect functional differences of EM fungi during intra- and interspecific plant competition for N and P.

EM diversity of beech roots increased in the presence of another beech (Appendix 1). This result indicates that a neighbouring tree with the same mycorrhizal type has a potential to increase the mycorrhizal community diversity and might enhance facilitation between tree individuals. Neighbour effects mediated by the mycorrhizal network need to be targeted by future research. As demonstrated in this work, stable and radioactive isotopes provide versatile, time-integrated measurements of nutrient and carbon fluxes. Future work could include pulse labelling of donator plant and measurements in proximate gainer plant to determine whether the nutrient and carbon fluxes occur only between trees with similar mycorrhizal types or are facilitative interactions possible also between fungi of different mycorrhizal types.

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Appendix 1

# Roots from beech (*Fagus sylvatica* L.) and ash (*Fraxinus excelsior* L.) differentially affect soil microorganisms and carbon dynamics

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## Highlights

- Rhizodeposition of beech reduced soil C by decreasing C use efficiency of bacteria
- More litter-derived carbon is channeled into higher trophic levels in soil planted with ash
- Bacteria and fungi form different energy channels in soil planted with beech
- In particular the roots of beech alter C dynamics in soil

**Abstract**

Knowledge on the influence of living roots on decomposition processes in soil is scarce but is needed to understand carbon dynamics in soil. We investigated the effect of dominant deciduous tree species of the Central European forest vegetation, European beech (*Fagus sylvatica* L.) and European ash (*Fraxinus excelsior* L.), on soil biota and carbon dynamics differentiating between root- and leaf litter-mediated effects. The influence of beech and ash saplings on carbon and nitrogen flow was investigated using leaf litter enriched in $^{13}C$ and $^{15}N$ in double-split-root rhizotrons planted with beech and ash saplings as well as a mixture of both tree species and a control without plants. Stable isotope and compound-specific fatty acid analysis ($^{13}C$-PLFA) were used to follow the incorporation of stable isotopes into microorganisms, soil animals and plants. Further, the bacterial community composition was analyzed using pyrosequencing of 15S rRNA gene amplicons. Although beech root biomass was significantly lower than that of ash only beech significantly decreased soil carbon and nitrogen concentrations after 475 days of incubation. In addition, beech significantly decreased microbial carbon use efficiency as indicated by higher specific respiration. Low soil pH probably increased specific respiration of bacteria and suggests that rhizodeposits of beech roots induced increased microbial respiration and therefore carbon loss from soil. Compared to beech, more litter carbon and nitrogen were channeled into higher trophic levels (Gamasida) in treatments with ash indicating higher amounts of litter-derived carbon to reach higher trophic levels under ash. Similar $\delta^{13}C$ signatures of bacteria and fine roots indicate that mainly bacteria incorporated root-derived carbon in beech rhizotrons. The results suggest that beech and ash differentially impact soil processes with beech more strongly affecting the belowground system via root exudates and associated changes in rhizosphere microorganisms and carbon dynamics than ash.

**Key words:** $^{13}C$, $^{15}N$, bacteria, carbon cycling, decomposition, fungi, nitrogen, soil food web

## 1. Introduction

Soils store twice as much carbon as plants and the atmosphere together thereby forming an important component of the global carbon cycle (Schlesinger and Andrews, 2000). However, the way carbon is processed and how carbon dynamics are controlled still is not well understood. Knowledge of controlling factors of the carbon flux from the entry into the soil until its release or sequestration is of significant importance, especially in face of global warming and climate disruption as a consequence of increased atmospheric $CO_2$ (McKinley et al., 2011).

In terrestrial ecosystems, 90% of the annual biomass produced by plants enters the dead organic matter pool forming the basis of the decomposer system in soil (Gessner et al., 2010). Plant carbon enters the soil via two pathways: dead organic matter (leaf litter and dead roots) and root exudates. Most studies suggest leaf litter quality as main factor explaining physical and chemical properties of soil systems, which in turn influence soil biota as drivers of decomposition processes (Reich et al., 2005; Jacob et al., 2009; Langenbruch et al., 2012). Litter quality strongly influences soil pH as calcium and magnesium of the litter compete with $H^+$ and $Al^{3+}$ for exchange sites on soil particle surfaces or organic matter (Reich et al., 2005). As a consequence, high pH often promotes higher microbial biomass resulting in higher soil respiration, mineralization and decomposition (Swift et al., 1979; Wardle, 1998). Low mineralization and decomposition rates are associated with high C-to-N ratios and high lignin contents as it is typical for recalcitrant litter. In contrast, Pollierer et al., (2007) highlighted that in temperate forests carbon does not enter the soil food web predominantly via litter but rather via roots. Rhizodeposits comprise labile exudates (e.g., sugars, amino acids and organic acids), but also complex molecules (e.g., polysaccharides, mucilage and proteins). Labile exudates control both community structure and activity of rhizosphere microorganisms (Paterson et al., 2009). Summarizing results of 95 plant $^{14}C$ labeling studies, Jones et al., (2004) estimated the loss of carbon by exudation to be equivalent to 5 - 10% of the net carbon fixed by plants and 25% of the carbon plants allocate to root growth. This supply of energy increases microbial biomass (Butler et al., 2004), acts as soil organic matter (SOM) priming agent (Bird et al., 2011) and alters the physical and chemical soil environment (Gregory, 2006). Microbial communities in rhizosphere and bulk soil are therefore responsible for root exudate-mediated changes in soil processes (Söderberg et al., 2004; Paterson et al., 2007). Since plant species differ in the quality and quantity of exudates (Jones et al., 2004), soil

carbon dynamics are likely affected by plant species identity and diversity (Grayston et al., 1998; Steinbeiss et al., 2008).

Next to individual effects of distinct plant species (Jacob et al. 2009), decomposition studies showed positive mixing effects (Gartner and Cardon, 2004; Hättenschwiler et al., 2005). Until today, however, studies investigating the influence of plant diversity on belowground dynamics in forests are scare (but see Meinen et al., 2009) and most often only consider the effect of aboveground plant residues (Hättenschwiler and Gasser, 2005; Jacob et al., 2009, 2010). To what extent belowground processes mediated by roots and root exudates affect soil organisms and thereby carbon dynamics remains largely unknown. This lack of knowledge is unfortunate as 60% of the terrestrial carbon is bound in forests and its contribution to global carbon cycling is of fundamental importance (McKinley et al., 2011).

To improve knowledge on carbon dynamics in forest soils from a root perspective we used the common temperate broad-leaved tree species European beech (*Fagus sylvatica* L.) and European ash (*Fraxinus excelsior* L.) to differentiate between general and species-specific effects of living roots on soil organisms and decomposition of litter material in soil. Beech is the dominant tree species in many Central European deciduous forests. Ash often is associated with beech forests and is expected to increase in dominance in a warmer and drier climate (Broadmeadow and Ray, 2005). Life history traits of beech and ash differ strongly, e.g. speed of growth, root morphology, litter quality, mycorrhizal association, and nutrient, water and light use efficiency (Grime et al., 1997; Emborg, 1998). Beech has higher specific root tip abundance, specific fine root surface area (SRA) and specific fine root length (SRL), whereas ash roots are characterized by higher mean fine root diameter (Meinen et al., 2009). Roots of beech are colonized by ectomycorrhizal (EM) fungi and those of ash by arbuscular mycorrhizal (AM) fungi which differ in nutrient acquisition strategies (Smith and Read, 2008). Beech tolerates soil pH from acid to highly alkaline, while ash is restricted to soils of high base saturation (Weber-Blaschke et al., 2002). Litter of beech at more acidic sites has high C-to-N ratio >50 and high lignin content, while ash litter is regarded as high quality litter due to its low C-to-N ratio of about 28 and low lignin content (Jacob et al., 2010).

For allowing access to the root system and to investigate interactions between both tree species, beech and ash saplings were planted into double split-root systems. The systems allowed dissecting root associated processes and belowground interactions between beech and ash. Carbon and nitrogen fluxes in soil were traced following the incorporation of $^{13}C$ and $^{15}N$

from labelled ash litter into soil, bacteria, fungi, soil animals and plants. Ash litter was used to follow the uptake of resources from high quality litter materials by beech and ash as compared to more recalcitrant soil resources.

We hypothesized that (1) beech and ash differentially affect the structure of the microbial community thereby modifying soil processes and plant nutrient capture. Differences in microbial community structure are expected to (2) result in differential decomposition of labeled ash litter and differential mobilization of nutrients from the litter. Further, we expected (3) modifications of the soil microorganisms community and soil processes to be most pronounced in the mixed treatment with both tree species present due to a complementary effect on soil microorganisms and soil processes.

## 2. Material and methods

### 2.1 Rhizotrons

Double split-root rhizotrons were used to separate root systems of two tree saplings into compartments with root strands of one individual sapling at each side and a shared root compartment in the centre where root strands of both tree saplings could interact (Fig. 1). We focused on the middle compartment where the two root strands grew together. The central compartment had a volume of 7.6 l and side compartments half the volume of 3.8 l. Rhizotrons were 90 cm high and 64 cm wide, and were built from anodized aluminum covered at the front with a 10-mm Perspex plate. They were tilted at 35° to direct roots growing along the Perspex plate. The Perspex plate was covered with black scrim to ensure that roots grow in darkness. Rhizotrons were divided into six soil depth sections (I-VI). Every soil depth contained four manipulation sites (MS), two in the centre and two at the sides (Fig. 1). The back side of the rhizotrons was equipped with a cooling system keeping the temperature at a constant level of 20ºC over the whole soil column. Climate conditions were set to 20°C air temperature, 70% relative air humidity and 10 h daylight in winter and 14 h in summer.

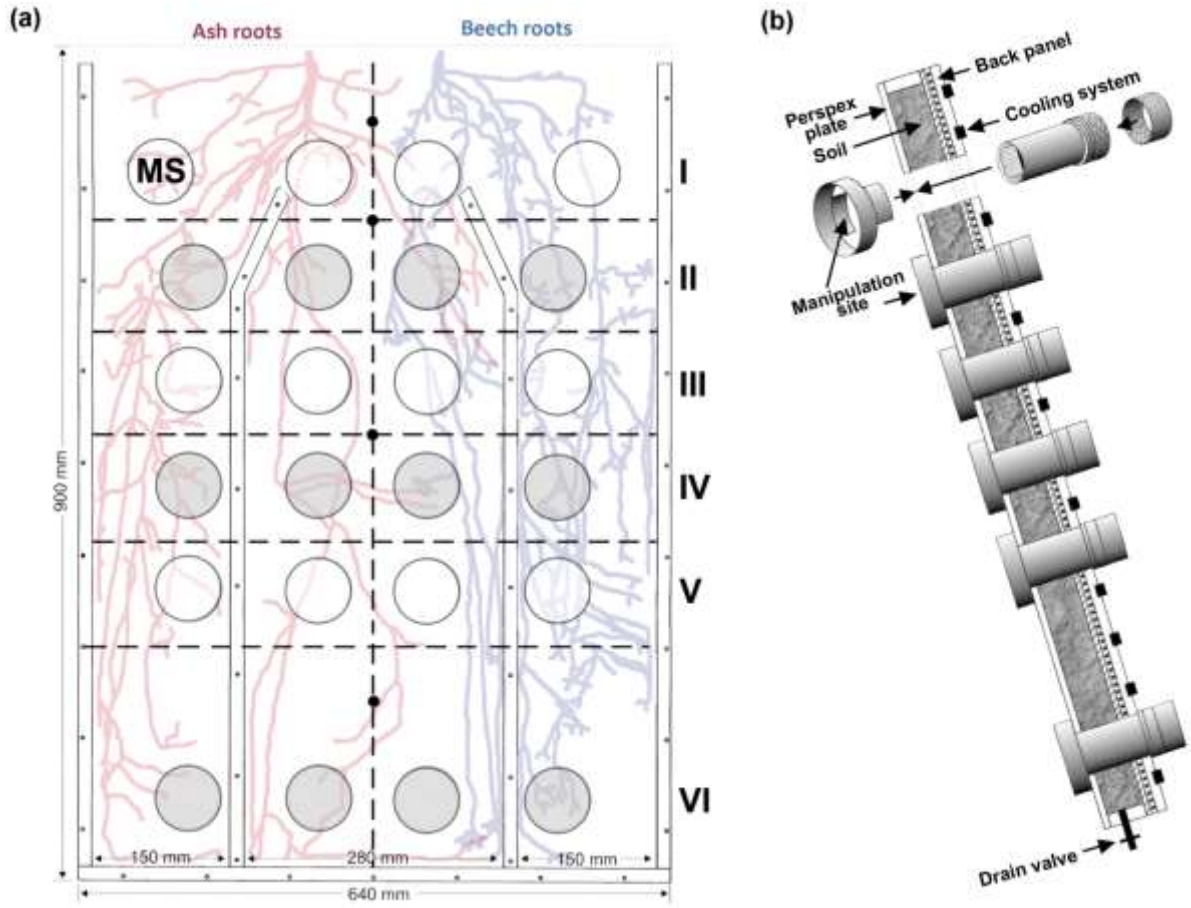


**Fig 1** | Schematic setup of the double split-root rhizotrons. (a) Front view of mixed species treatments with ash (red) and beech (blue) roots interacting in the central compartment. Circles represent manipulation sites (MS) with soil (open circles) or a soil-litter mixture (shaded circles). Numbers indicate soil depths (I-VI). Black dots along central dashed line refer to the position of temperature sensors. Dashed lines mark the sampling raster in which soil samples were separated. (b) Side view of the double split-root rhizotron and assembly of the MS. Tubes inside the MS can be drawn completely to fill it with litter and soil. A cooling system is installed at the back panel and is flooded with distilled water. A valve allowed drainage of the rhizotrons.

The tree saplings were illuminated (EYE Lighting, Clean Ace, Mentor, OH, USA) ensuring a minimum PPFD of 200 ± 10 µmol $m^{-2}$ $s^{-1}$ from June 2009 to October 2010. The experiment lasted for 475 days i.e., plants were harvested after the second season.

### 2.1.1 Soil and plants

The soil was taken from a mixed temperate broadleaf forest dominated by *F. sylvatica*, *F. excelsior* and *Tilia cordata* in Central Germany (Hainich forest, 51°04' N 10°30' E, about 350 m a.s.l) from a depth of 0 - 10 cm after removing the litter. The soil type was a Stagnic

**Table 1** | Soil characteristics (means ± 1 SE) at the start of the experiment.

| **Soil parameter** | | | |
|---|---|---|---|
| pH | 4.56 | ± | 0.03 |
| CEC [$\mu mol_c\ g^{-1}$ dry weight] | 191.70 | ± | 11.80 |
| Base saturation [%] | 22.90 | ± | 1.30 |
| $N\text{-}NO_3^-$ [mg $kg^{-1}$ dry weight] | 4.89 | ± | 0.20 |
| $N\text{-}NH_4^+$ [mg $kg^{-1}$ dry weight] | 6.00 | ± | 0.22 |
| $C_{org}$ [g $kg^{-1}$ dry weight] | 19.20 | ± | 0.30 |
| $N_{total}$ [g $kg^{-1}$ dry weight] | 1.64 | ± | 0.01 |
| C/N ratio [g $g^{-1}$] | 11.70 | ± | 0.14 |
| $C_{mic}$ [µg C $g^{-1}$ dry weight] | 382.80 | ± | 14.60 |

Luvisol (IUSS Working Group WRB 2007; 1.8% sand, 80.2% silt and 18.1% clay) and free of carbonate (<0.02% of total carbon) with a pH ($H_2O$) of 4.56 ± 0.03 and a gravimetric water content at date of sampling of 22.7%. Initial total carbon amounted to 19.2 ± 0.3 g $kg^{-1}$ dry weight, initial total nitrogen averaged 1.56 ± 0.01 g $kg^{-1}$ dry weight and base saturation was 22.9 ± 1.3% (Table 1). Each rhizotron was filled with 15.2 l of sieved soil (1 cm mesh) containing natural microflora and soil fauna. Volumetric soil water content was monitored three times a week with a TDR measurement device (Trime-FM, IMKO, Ettlingen, Germany), and kept at constant level by adding distilled water. Soil temperature was measured with NTC thermistors (Epcos, Munich, Germany), arranged vertically in the centre of the rhizotrons, at soil depths of 8, 20, 42.5 and 70.5 cm and in 2 cm distance of the Perspex plate. Data were recorded in 15-min intervals with a CR1000 data logger (combined with two AM416 Relay Multiplexer, Campbell Scientific Inc., Utah, USA).

In spring 2009, beech (*F. sylvatica*) and ash (*F. excelsior*) saplings with comparable root biomass were excavated in the Hainich forest with intact soil cores to preserve the root and 15.4±1.2 cm for beech and ash saplings, respectively. At the start of the experiment, ash had significantly higher fine root biomass than beech, but tree species did not differ significantly in total root and total aboveground biomass (Table 2). Before planting, the soil material adhering to the root systems was removed by watering. The remaining soil-water mixture was used to equilibrate microbial communities in soil.

**Table 2** | T-test table of *T*- and *P*-values and means ± 1 SE of plant biomass $g^{-1}$ dry weight of beech and ash saplings at the start of the experiment (n=5).

| | | | Initial Biomass | |
|---|---|---|---|---|
| **Biomass** | *T* | *P* | Beech | Ash |
| Total | 0.15 | 0.7122 | 2.04 ± 0.46 | 2.13 ± 0.22 |
| Total aboveground | 0.01 | 0.9294 | 1.26 ± 0.27 | 1.25 ± 0.15 |
| Total belowground | 0.81 | 0.3933 | 0.78 ± 0.20 | 0.88 ± 0.08 |
| Shoots | 0.27 | 0.6190 | 0.88 ± 0.22 | 0.74 ± 0.09 |
| Leaves | 2.49 | 0.1530 | 0.38 ± 0.05 | 0.52 ± 0.07 |
| Fine roots | **6.49** | **0.0343** | 0.16 ± 0.05 | 0.41 ± 0.08 |
| Coarse roots | 0.08 | 0.7866 | 0.64 ± 0.17 | 0.56 ± 0.06 |

Fifty-three days after planting, 1.5 g labelled ash litter was added into MS at every second soil depth (II, IV, VI; Fig. 1). Prior to adding ash leaves (air dried, crushed to pieces < 1 cm) were mixed with 40 g soil (air dried). The litter was labelled with $^{13}C$ and $^{15}N$ by incubating ash trees in a green house for one vegetation period with the $CO_2$ concentration in air elevated by adding $^{13}CO_2$ (1,200 ppm) and by watering the soil with nutrient solution containing and 0.1 mM $^{15}NO_3{}^{15}NH_4$ (both 99 atom %; Euriso-top, Saint-Aubin, Essonne, France). Further, the solution contained 0.6 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.01 mM $FeCl_3$, 0.4 mM $K_3PO_4$, 1.8 µM $MnSO_4$, 0.064 µM CuCl, 0.15 µM $ZnCl_2$, 0.1 µM $MoO_3$, 5 mM $NO_3NH_4$ and 0.01 mM $H_3BO_3$,. The stable isotope signature of the ash litter was 146.8 ± 0.3‰ for $\delta^{13}C$ and 13,139 ± 59‰ for $\delta^{15}N$ (Table 3).

### 2.2 Experimental design

The experiment was set up in a two-factorial design with the factors beech and ash (absence: "-" and presence: "+"), resulting in the following treatments with four replicates each: (a) two beech saplings (BB), (b) two ash saplings (AA), (c) a mixture with one beech and one ash sapling (BA or AB, depending on target tree species), and (d) an unplanted control (Co), resulting in rhizotrons without (B-: Co and AA) and with beech (B+: BB and BA), as well as rhizotrons without (A-: Co and BB) and with ash (A+: AA and AB).

### 2.3 Sampling

After 475 days rhizotrons were harvested. They were opened in horizontal position and a sampling grid was used to identify locations for sampling i.e., at MS and the surrounding of these sites (SS) (Fig. 1). Samples from the depth layers II, III, IV and V of the central compartment were analyzed. Further, as we were not interested in effects of soil depth we pooled the data from the four layers. In addition to soil samples, plant shoots and roots from each of the soil layers were taken for measuring plant biomass.

**Table 3** | Isotopic signatures of the used soil, labeled ash litter and of the soil-litter-mixture in manipulation sites in rhizotrons at the start of the experiment and after 422 days (means ± 1 SE).

| | Start | | | End | |
|---|---|---|---|---|---|
| | **Soil** | **Litter** | **Soil-litter mixture** | **Soil-litter mixture** | **Difference * [%]** |
| **$\delta^{13}C$ [‰]** | -26.20±0.10 | 146.80±0.32 | 69.00±0.60 | -17.44±1.86 | 88.25 |
| **$\delta^{15}N$ [‰]** | 1.60±0.16 | 13139.30±59.10 | 6153.80±0.40 | 577.38±124.88 | 81.23 |
| **C [%]** | 1.92±0.03 | 36.05±0.09 | 5.93±0.05 | 1.94±0.06 | 65.34 |
| **N [%]** | 0.16±0.00 | 1.85±0.01 | 0.40±0.00 | 0.18±0.004 | 54.82 |
| **C/N** | 11.70±0.10 | 19.50±0.10 | 14.90±0.10 | 10.98±0.12 | 15.33 |

* Differences between isotopic signatures from the start and the end of the experiment base on values of soil-litter mixtures and were calculated overall treatments (n=16) and were related to the natural isotopic signatures of beech and ash litter.

#### 2.3.1 Plants

At harvest shoot length and root collar diameter of saplings was measured. Roots were separated from soil, washed and cleaned from adhering soil particles. To obtain overall plant biomass fine root biomass estimated from MS for mycorrhizal analysis were combined with plant biomass data from SS. Whenever possible three intact root strands of ca. 7 cm length from each tree species per compartment and soil depths were taken and digitalised on a flat-bed scanner for image analysis carried out using WinRhizo 2005c software (Régent Instruments Inc., Québec, QC, Canada) to determine specific fine root area (SRA; $cm^2\ g^{-1}$ dry matter), specific fine root length (SRL; cm $g^{-1}$ dry matter) and total fine root surface. Thereafter, samples were oven-dried (70°C, 48 h), weighed and milled for measurement of organic carbon ($C_{org}$), total nitrogen ($N_{total}$) as well as $\delta^{13}C$ and $\delta^{15}N$ signatures (Delta C, Finnigan MAT, Bremen, Germany).

### 2.3.2 Mycorrhiza

Colonization of roots at MS by mycorrhiza-forming fungi was determined. Fine roots were stored in Falcon tubes with moist tissue paper at 4°C until analysis. Fine roots of beech were analyzed with a stereomicroscope (Leica M205 FA, Leica Microsystems, Wetzlar, Germany). The percentage of EM fungi colonization was calculated using the following equation:

$$\text{EM fungi colonization [\%]} = \left(\frac{n\ mycorrhizal\ root\ tips}{n\ vital\ root\ tips}\right) \times 100 \qquad \text{(eq. 1)}$$

Fine roots of ash were stored in 70% EtOH at room temperature. For determining the colonization by AM fungi roots were stained with lactophenole-blue (Schmitz et al., 1991) and stored at room temperature in 50% glycerol until microscopic inspection at 200x magnification. AM fungi colonization was calculated with the magnified intersection method of McGonigle et al., (1990) using a 10x10 grid. The abundance of vesicles, arbuscles and hyphae was calculated as percentage of mycorrhizal structures of the total number of intersections. The percentage of vesicles was taken as relative colonization rate of AM fungi and used for further calculations.

### 2.3.4 Soil properties

Soil pH was measured in a suspension of 10 g soil and 25 ml $H_2O$ with a Vario pH meter (WTW GmbH, Weilheim, Germany). Soil water content was measured gravimetrically after drying at 105°C for 24 h. Nitrate and ammonium concentrations were measured by extracting soil samples in 0.5 M $K_2SO_4$ solution (1:3 wet soil mass-to-solution ratio). Samples were shaken for 1 h and filtered through Sartorius folded filters (Sartorius Stedim, Aubagne, France). Nitrate and ammonium concentrations of filtered extracts were analyzed using continuous flow injection colorimetry ($SAN^+$ Continuous Flow Analyzer, Skalar Instruments, Breda, The Netherlands). Nitrate was determined by copper cadmium reduction method (ISO method 13395) and ammonium was quantified by Berthelot reaction method (ISO method 11732). $C_{org}$, $N_{total}$ as well as $\delta^{13}C$ and $\delta^{15}N$ values were measured after grinding soil samples with a disc mill. Samples were analyzed with a coupled system consisting of an elemental analyzer (NA 1500, Carlo Erba, Mailand) and a mass spectrometer (Delta C, Finnigan MAT, Bremen, Germany).

### 2.3.5 Microbial respiration

Basal respiration (BAS), microbial biomass ($C_{mic}$), and specific respiration ($qO_2$) were measured by substrate-induced respiration (SIR) i.e., the respiratory response of microorganisms to glucose (Anderson and Domsch, 1978). Before measurement, roots were removed and soil samples were sieved (2 mm). Measurements were done using an automated $O_2$ microcompensation system (Scheu, 1992). BAS of microorganisms reflected their averaged oxygen consumption rate without the addition of glucose within 10-30 h after attachment of the samples to the analysis system. Subsequently, 4 mg glucose $g^{-1}$ soil dry weight was added as aqueous solution to the soil samples. The mean of the three lowest hourly measurements within the first 10 h was taken as the maximum initial respiratory response (MIRR). $C_{mic}$ (µg C $g^{-1}$) was calculated as 38 x MIRR (µl $O_2$ $g^{-1}$ soil dry weight $h^{-1}$) according to Beck et al., (1997). Microbial specific respiration $qO_2$ (µl $O_2$ $mg^{-1}$ $C_{mic}$ $h^{-1}$) was calculated as BAS/$C_{mic}$.

### 2.3.6 Fatty acid analysis

Before extraction of lipids, soil samples were sieved (2 mm) and root and litter pieces were removed. Lipid extraction followed Frostegård et al., (1991). Briefly, 4 g soil (wet weight) was mixed with 18.5 ml Bligh & Dyer solution and shaken for 2 h(Bligh and Dyer, 1959). Subsequently, samples were centrifuged at 2,500 rpm for 10 min at 8°C. Supernatants were transferred to new tubes. The remaining pellet was washed with 5 ml of the Bligh & Dyer solution and centrifuged as described above. The supernatants were combined and 6.2 ml chloroform and 6.2 ml citrate buffer were added. Two ml of the lipid containing lower phase was transferred to a new tube. The organic phase was evaporated at 40°C for 40 min. Columns containing silic acid fractionated the lipid material into phospholipids by adding methanol. The phospholipid-methanol solution was evaporated at 40°C for 90 min. Each sample was dissolved in 1 ml methanol-toluene-solvent (1:1) and 30 µl internal Standard (5.77 mg methylnondecanoat in 25 ml isooctane) was added. Basic methanolysis of lipids was conducted in 1 ml 0.2 M methanolic KOH (2.8 g KOH in 250 ml methanol) incubated at 37°C for 15 min. Afterwards, 2 ml hexane, 0.3 ml acetic acid and 2 ml deionized water were added, vortexed and centrifuged as described above. The upper phase was transferred to new tubes and evaporated at 40°C for 45 min. The remaining extract was solved with 100 µl isooctane and filled into 1.5 ml vials for analysis. Bacterial biomass was estimated using the following PLFAs: a15:0, i15:0, i16:0, 16:1ω7, i17:0, cy17:0 and cy19:0; the PLFA 18:2ω6,9 was used

as fungal biomarker (Ruess and Chamberlain, 2010). A gas-chromatography-combustion-isotope-ratio-monitoring-mass spectrometer (GC-C-IRM-MS) using Thermo Finnigan Trace GC coupled via a GP interface to a Delta Plus mass spectrometer (Finnigan, Bremen, Germany) was used to determine the isotopic composition of individual PLFAs. Fatty acid identification was verified by GC-MS using a Varian CP-3800 chromatograph coupled to a 1200L mass spectrometer and a fused silica column (Phenomenex Zebron ZB-5MS, 30 m, 0.25 µm film thickness, ID 0.32 mm) and helium as carrier gas.

### 2.3.7 Pyrosequencing

DNA and RNA were co-isolated from 2 g soil using the RNA PowerSoil™ Total RNA Isolation Kit and DNA Elution Accessory Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). Residual DNA contaminations in RNA extracts were removed using the TURBO DNA-*free*™ Kit (Ambion Applied Biosystems, Darmstadt, Germany). RNA was concentrated using the RNeasy MiniElute Kit (QIAGEN, Hilden, Germany). The nucleic acid concentration was estimated using a NanoDrop ND-1000 spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany).

The V2-V3 region of the 16S rRNA was reverse transcribed using the SuperScript™ III reverse transcriptase (Invitrogen, Karlsruhe, Germany). As template 100 ng of the DNA-free RNA were applied. The resulting cDNA as well as the extracted DNA was amplified in triplicate using the Phusion® Hot Start High-Fidelity DNA polymerase (FINNZYMES, Espoo, Finland) as described by Nacke et al., (2011).

The following barcoded primer set was used for reverse transcription and amplification, containing the Roche 454 pyrosequencing adaptors (underlined): V2for 5'-CTATGCGCCTTGCCAGCCCGCTCAGAGTGGCGGACGGGTGAGTAA-3' and V3rev 5'-CGTATCGCCTCCCTCGCGCCATCAGCGTATTACCGCGGCTGCTG-3'

modified from (Schmalenberger et al., 2001).

The PCR products were treated and purified as described by Nacke et al., (2011). All kits were used as described in the manufacturer´s instructions. The Göttingen Genomics Laboratory determined the sequences of the partial 16S rRNA genes using a Roche GS-FLX 454 pyrosequencer (Roche, Mannheim, Germany) according to the manufacturer´s instructions for amplicon sequencing. Sequences shorter than 300 bp were removed from the dataset.

To minimize the bias introduced by pyrosequencing due to decreasing read precision at the end of the reads denoising was carried out using Denoiser 0.91 (Reeder and Knight, 2010). OTU determination was performed using uclust OTU picker 1.2.22q (Edgar, 2010) at genetic divergence of 3%, 5% and 20% according to Schloss and Handelsman (2005). The resulting datasets have been deposited in the GenBank short-read archive under accession number SRA050002.

### 2.3.8 Soil animals

Soil not needed for other analysis was taken to extract soil animals by heat (Kempson et al., 1963). Animals were conserved in saturated NaCl solution and kept at -10°C until analysis. The gamasid mite *Hypoaspis aculeifer* (G. Canestrini, 1884) was taken for stable isotope analysis as it occurred in sufficient numbers for the analysis. Twenty adult mites were weighed into tin capsules and dried at 40°C for 24 h. Samples were analyzed as described above.

## 2.4 Statistical analysis

Two-way ANOVA was used to test for main effects of beech (B- and B+), ash (A- and A+) and their interactions with data of the four soil depths pooled. To detect differences in plant biomass and mycorrhizal colonization contrasts were calculated using pairwise *t*-test to account for dependence in mixed rhizotrons. U-Test was used for analyzing the number of root tips. Treatments in beech-only rhizotrons (BB) were compared to ash-only (AA) and beech-ash mixture (BA). Ash (AA) was also compared with beech-ash mixture (AB). Statistical analyzes were done using SAS 9.2 (SAS Institute; Cary, NC, USA).

Discriminant function analysis (DFA) was calculated to analyze fatty acid patterns combined with microbial respiration and soil chemical data. Differences of the bacterial composition in beech and ash rhizotrons and the control were calculated using multi-dimensional scaling (MDS) to reduce dimensions in the dataset. DFA and MDS were calculated using STATISTICA 7.0 for Windows (StatSoft, Tulsa, USA, 2001).

Means were compared using Tukey's Honestly Significant Difference test ($P < 0.05$). Data were log- or arcsine-square root transformed (percentages) to improve homogeneity of variance. Means given in text and tables are based on non-transformed data.

## 3. Results

### 3.1 Plants and mycorrhizae

After 475 days, total biomass of tree saplings in BB rhizotrons was significantly lower than in AA and BA rhizotrons (Table 4). Fine and coarse root biomass were significantly lower in BB rhizotrons compared to that of saplings in AA (-69%) and BA rhizotrons (-62%) resulting in significantly lower total root biomass. Total biomass, total root biomass and coarse root biomass of saplings in mixtures exceeded that of saplings in monocultures, but this increase was only significant for beech (60%, 62%, 70%, respectively); biomass of ash saplings in mixture increased by 11%, 17% and 23%, respectively.

$\delta^{13}C$ and $\delta^{15}N$ signatures in fine roots were significantly lower in BB than those in AA rhizotrons (Table 4). SRA and SRL did not differ significantly between tree species but tended to be higher in beech (BB vs AA: + 6% and +68%, respectively), especially in the mixture (BA vs AB: +24% and 79%, respectively. Generally, fine root tips of tree saplings increased in mixed rhizotrons, especially beech in mixed rhizotrons had a significantly higher number of root tips than beech in monoculture by +89% compared to ash in mixed rhizotrons and ash in monoculture by +54%. Mycorrhizal colonization of roots of beech in BB rhizotrons was significantly lower than that of roots of ash in AA rhizotrons, however, as beech and ash are colonized by different types of mycorrhiza the differences have to be interpreted with caution. Beech did not influence the colonization rate of ash by arbuscular mycorrhiza (AA vs AB; + 2 %), whereas ash increased the colonization of beech by ectomycorrhiza (BB vs BA; + 45 %) although the effect was not significant (Table 4).

### 3.2 Soil properties

In general, the studied soil properties were strongly affected by beech and not by ash with interactions between tree species also being not significant (Table 5). Soil pH was significantly lower in B+ (4.54 ± 0.08) than in B- rhizotrons (4.80 ± 0.06). In presence of beech $C_{org}$ and $N_{total}$ were significantly decreased by -7% and -6%, respectively, but $NO_3^-$ and $NH_4^+$ concentrations remained unaffected. Further, $\delta^{13}C$ and $\delta^{15}N$ of bulk soil were significantly lower in B+ (-24.46 ± 0.32‰ and 127.04 ± 19.95‰, respectively) compared to B- rhizotrons (-22.24 ± 0.78‰ and 265.25 ± 48.79‰, respectively). Generally, after 422 days of litter incubation, the signatures of $\delta^{13}C$ and $\delta^{15}N$ within the soil-litter-mixtures decreased strongly by 88% and 81% respectively (Table 3).

**Table 4** | Contrasts calculated with *t*-test for all plant parameters except for number of fine root tips (*U*-test) and means ± 1 SE for differences in plant parameters between individual trees in rhizotrons after 475 days.

| | **Pure beech (BB) vs. pure ash (AA)** | | **Pure beech (BB) vs. beech in mixture (BA)** | | **Pure ash (AA) vs. ash in mixture (AB)** | | **BB** | **AA** | **BA** | **AB** |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***t*** | ***P*** | ***t*** | ***P*** | ***t*** | ***P*** | | | | |
| **Biomass [g dry weight] plant** | | | | | | | | | | |
| Total | **8.82** | **0.0117** | **6.39** | **0.0266** | 0.00 | 0.9518 | 4.52 ± 0.79 | 12.08 ± 1.69 | 11.33 ± 2.03 | 13.50 ± 2.33 |
| Total aboveground | 4.20 | 0.0629 | 2.91 | 0.1138 | 1.11 | 0.3128 | 1.85 ± 0.38 | 3.34 ± 0.86 | 4.29 ± 1.01 | 3.03 ± 0.43 |
| Total root | **9.52** | **0.0094** | **6.96** | **0.0217** | 0.28 | 0.6035 | 2.67 ± 0.50 | 8.74 ± 1.08 | 7.04 ± 1.08 | 10.47 ± 2.15 |
| Shoot | 1.24 | 0.2876 | 3.43 | 0.0889 | 0.12 | 0.7300 | 1.38 ± 0.25 | 1.78 ± 0.32 | 3.20 ± 0.73 | 2.64 ± 0.41 |
| Leaves | **5.14** | **0.0426** | 0.53 | 0.4809 | **7.50** | **0.0180** | 0.46 ± 0.14 | 1.56 ± 0.56 | 1.08 ± 0.34 | 0.39 ± 0.22 |
| Fine roots | **9.14** | **0.0106** | 4.60 | 0.0532 | 0.09 | 0.7669 | 0.78 ± 0.18 | 2.38 ± 0.30 | 1.80 ± 0.27 | 2.27 ± 0.44 |
| Coarse roots | **7.95** | **0.0154** | **6.50** | **0.0255** | 0.59 | 0.4557 | 1.89 ± 0.35 | 6.36 ± 0.87 | 5.24 ± 0.82 | 8.21 ± 1.91 |
| **$\delta^{13}C$ [‰] Plant fractions** | | | | | | | | | | |
| Shoot | **5.14** | **0.0426** | **7.00** | **0.0214** | 2.12 | 0.1708 | -29.09 ± 0.32 | -28.07 ± 0.28 | -27.90 ± 0.22 | -27.40 ± 0.26 |
| Leave | 0.30 | 0.5955 | 0.25 | 0.6287 | 0.75 | 0.4029 | -29.62 ± 0.56 | -29.26 ± 0.27 | -29.29 ± 0.44 | -29.83 ± 0.20 |
| Fine roots | **8.27** | **0.0139** | 0.04 | 0.8402 | 0.01 | 0.9395 | -27.64 ± 0.34 | -25.60 ± 0.85 | -27.49 ± 0.19 | -25.56 ± 0.23 |
| Coarse roots | **12.86** | **0.0037** | 2.78 | 0.1215 | 0.06 | 0.8162 | -28.35 ± 0.31 | -25.74 ± 0.76 | -27.15 ± 0.31 | -25.92 ± 0.32 |
| **$\delta^{15}N$ [‰] Plant fractions** | | | | | | | | | | |
| Shoot | 0.87 | 0.3701 | 0.07 | 0.8018 | 2.15 | 0.1682 | 171.27 ± 30.67 | 260.05 ± 66.16 | 154.54 ± 18.34 | 154.40 ± 26.76 |
| Leaves | **5.34** | **0.0394** | 0.55 | 0.4741 | 1.98 | 0.1853 | 192.42 ± 32.67 | 316.50 ± 43.37 | 166.67 ± 23.49 | 228.28 ± 15.10 |
| Fine roots | **4.77** | **0.0496** | 1.35 | 0.2674 | 4.07 | 0.0666 | 209.02 ± 41.75 | 396.07 ± 99.34 | 148.85 ± 17.63 | 214.48 ± 22.80 |
| Coarse roots | **9.34** | **0.0100** | 0.10 | 0.7630 | 2.81 | 0.1196 | 193.66 ± 27.78 | 390.78 ± 78.87 | 178.50 ± 12.60 | 257.86 ± 19.23 |
| **SRA [$cm^2\ g^{-1}$]** | | | | | | | | | | |
| Fine roots | 0.23 | 0.6385 | 0.05 | 0.8271 | 0.78 | 0.3950 | 485.16 ± 15.36 | 456.49 ± 42.70 | 509.00 ± 54.07 | 410.65 ± 64.00 |
| **SRL [$cm\ g^{-1}$]** | | | | | | | | | | |
| Fine roots | 2.89 | 0.1150 | 0.50 | 0.4947 | 0.20 | 0.6596 | 2374.80 ± 221.17 | 1414.42 ± 168.82 | 3235.44 ± 848.14 | 1810.83 ± 450.85 |
| **Fine root tips** | | | | | | | | | | |
| Total number | -0.48 | 0.9970 | **-13.16** | **0.00010** | 2.13 | 0.1750 | 1623.50 ± 230.01 | 2299.00 ± 419.58 | 3072.50 ± 207.37 | 3543.75 ± 107.79 |
| **Mycorrhiza [%]** | | | | | | | | | | |
| Colonization rate | **27.50** | **0.0002** | 3.07 | 0.1053 | 0.04 | 0.8481 | 37.81 ± 8.58 | 81.82 ± 5.17 | 54.80 ± 6.51 | 83.54 ± 2.87 |

**Table 5** | ANOVA table of *F*- and *P*-values on the effects of beech and ash and interactions between both tree species in rhizotrons as well as means ± 1SE.

| | Beech | | Ash | | Beech × Ash | | Beech absent (B-) | | Beech present (B+) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***F*** | ***P*** | ***F*** | ***P*** | ***F*** | ***P*** | **Ash absent (A-)** (Control) | **Ash present (A+)** (Ash) | **Ash absent (A-)** (Beech) | **Ash present (A+)** (Mixture) |
| **Soil data** | | | | | | | | | | |
| pH ($H_2O$) | **5.77** | **0.0334** | 0.11 | 0.7436 | 0.02 | 0.8944 | 4.78 ± 0.12 | 4.83 ± 0.05 | 4.53 ± 0.14 | 4.55 ± 0.11 |
| N-$NO_3^-$ [mg $kg^{-1}$ dry | 1.00 | 0.3387 | 0.04 | 0.8532 | 0.62 | 0.4487 | 41.31 ± 4.96 | 42.88 ± 3.20 | 39.32 ± 3.83 | 35.14 ± 5.93 |
| N-$NH_4^+$[mg $kg^{-1}$ dry | 0.01 | 0.9422 | 0.41 | 0.5360 | 0.38 | 0.5477 | 2.46 ± 0.93 | 1.47 ± 0.63 | 1.88 ± 0.72 | 1.86 ± 0.69 |
| $C_{org}$ [mg $kg^{-1}$ dry weight] | **15.02** | **0.0022** | 0.08 | 0.7829 | 0.02 | 0.8980 | 1.89 ± 0.04 | 1.91 ± 0.05 | 1.76 ± 0.03 | 1.77 ± 0.02 |
| $N_{total}$ [mg $kg^{-1}$ dry weight] | **7.82** | **0.0162** | 0.24 | 0.6297 | 0.00 | 0.9687 | 0.18 ± 0.00 | 0.17 ± 0.00 | 0.17 ± 0.00 | 0.16 ± 0.00 |
| $\delta^{13}C$ soil [‰] | **7.54** | **0.0177** | 1.73 | 0.2129 | 1.40 | 0.2604 | -23.27 ± 0.58 | -21.21 ± 1.35 | -24.51 ± 0.57 | -24.41 ± 0.40 |
| $\delta^{15}N$ soil [‰] | **7.42** | **0.0185** | 0.83 | 0.3816 | 0.31 | 0.5907 | 212.18 ± 55.44 | 318.33 ± 78.47 | 126.29 ± 37.64 | 127.79 ± 20.99 |
| C/N | 0.56 | 0.4677 | 0.98 | 0.3406 | 0.00 | 0.9932 | 10.78 ± 0.24 | 10.94 ± 0.08 | 10.66 ± 0.16 | 10.82 ± 0.14 |
| **Microbial respiration** | | | | | | | | | | |
| BAS [µl $O_2$ $h^{-1}$ g] | 4.04 | 0.0674 | 0.09 | 0.7674 | 0.19 | 0.6701 | 1.18 ± 0.09 | 1.18 ± 0.05 | 1.41 ± 0.07 | 1.36 ± 0.15 |
| $C_{mic}$ [µg C $g^{-1}$] | 0.03 | 0.8643 | 0.48 | 0.5019 | 0.40 | 0.5365 | 150.03 ± 13.65 | 134.32 ± 5.93 | 139.79 ± 6.62 | 140.86 ± 13.38 |
| $qO_2$ [µl $O_2$ $mg^{-1}$ $C_{mic}$ $h^{-1}$] | **9.00** | **0.0111** | 0.14 | 0.7178 | 1.59 | 0.2311 | 0.008 ± 0.001 | 0.009 ± 0.000 | 0.010 ± 0.001 | 0.010 ± 0.001 |
| **PLFA [nmol $g^{-1}$dry weight]** | | | | | | | | | | |
| Total | 0.75 | 0.4025 | 0.00 | 0.9619 | 1.11 | 0.3130 | 7.22 ± 1.32 | 6.03 ± 1.36 | 6.57 ± 0.55 | 8.19 ± 0.97 |
| Bacteria | 0.53 | 0.4801 | 0.01 | 0.9377 | 1.05 | 0.3262 | 6.95 ± 1.20 | 5.85 ± 1.29 | 6.25 ± 0.52 | 7.66 ± 0.95 |
| Fungi | 3.36 | 0.0916 | 0.18 | 0.6757 | 1.20 | 0.2955 | 0.27 ± 0.16 | 0.18 ± 0.07 | 0.33 ± 0.05 | 0.53 ± 0.15 |
| Fungi/Bacteria | **5.17** | **0.0422** | 0.33 | 0.5755 | 0.85 | 0.3752 | 0.032 ± 0.017 | 0.026 ± 0.010 | 0.050 ± 0.008 | 0.073 ± 0.019 |
| **PLFA $\delta^{13}C$ [‰]** | | | | | | | | | | |
| Total | 2.43 | 0.1454 | 1.40 | 0.2590 | 0.30 | 0.5944 | -22.80 ± 2.37 | -21.49 ± 2.09 | -27.14 ± 0.60 | -23.55 ± 2.51 |
| Bacteria | 2.01 | 0.1818 | 0.49 | 0.4960 | 0.51 | 0.4871 | -24.38 ± 1.47 | -24.43 ± 1.05 | -27.25 ± 0.45 | -25.31 ± 1.89 |
| Fungi | **7.48** | **0.0181** | 0.08 | 0.7807 | 0.16 | 0.6941 | -21.01 ± 6.61 | -17.06 ± 4.53 | -31.59 ± 0.92 | -28.27 ± 4.01 |
| **Gamasid mites** | | | | | | | | | | |
| **$\delta^{13}C$ [‰]** | **20.59** | **0.0008** | **159.43** | **<.0001** | **7.80** | **0.0175** | -23.37 ± 0.86 | -13.89 ± 0.31 | -20.19 ± 1.40 | -8.78 ± 0.43 |
| **$\delta^{15}N$ [‰]** | **25.75** | **0.0004** | **148.88** | **<.0001** | **11.93** | **0.0054** | 130.14 ± 23.08 | 713.33 ± 43.37 | 339.07 ± 37.35 | 1121.26 ± 26.97 |

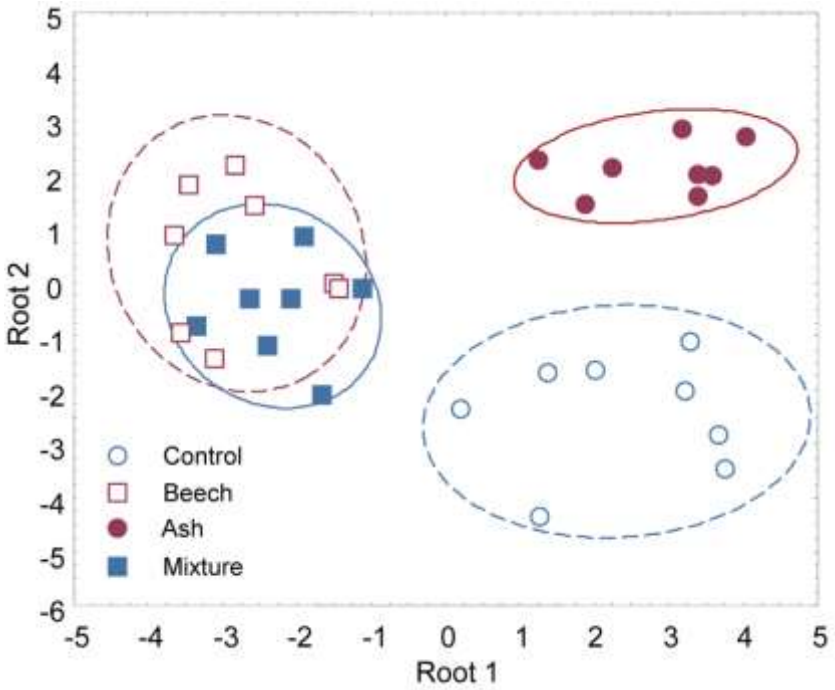


**Fig 2** | Discriminant functional analysis (DFA) of microbial PLFAs, microbial respiration and soil properties in rhizotrons without trees (control), beech saplings, ash saplings and a mixture of both tree species. Wilks' Lambda: 0.016480, F (54,33) = 1.85, *P* = 0.0296. Ellipses represent confidence intervals at *P* = 0.05.

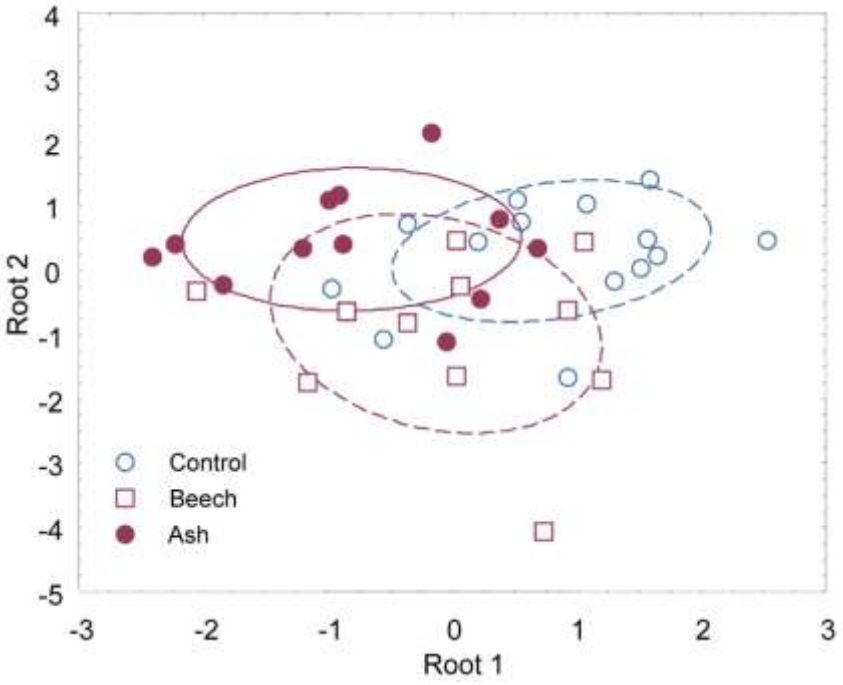


**Fig 3** | Discriminat function analysis (DFA) of bacterial phyla based on pyrosequencing of 16S rRNA in rhizotrons without trees (control) and with beech and ash saplings after reducing data to 6 dimensions by multidimensional scaling (MDS). Wilks' Lambda: 0.499576; *F* (12,60) = 2.07; *P* = 0.0325. Ellipses represent confidence intervals at *P* = 0.05.

### 3.3 Microorganisms

$C_{mic}$ was not significantly affected by tree species and averaged over all treatments 141.25 ± 4.93 µg C $g^{-1}$. However, $qO_2$ was significantly higher in B+ (0.0101 ± 0.003 µl $O_2$ $mg^{-1}$ $C_{mic}$ $h^{-1}$) than B- rhizotrons (-16%, Table 5), which was due to marginally higher BAS in B+ (1.39 ± 0.08 µl $O_2$ $h^{-1}$ $g^{-1}$) compared to B- rhizotrons (-15%).

The ratio of fungal-to-bacterial marker PLFAs was significantly higher in B+ (0.061 ± 0.007) than in B- rhizotrons (-53%) as the fungal biomass was higher in B+ (0.43 ± 0.08 nmol $g^{-1}$dry weight) than in B- rhizotrons (-47%), whereas bacterial biomass remained unaffected.

**Table 6** | Summary of input variables of the discriminant function analysis (DFA) of the composition of PLFA of the microbial community, soil properties and microbial respiration.

| | | Wilks' Lambda | F (3,11) | *P*-level |
|---|---|---|---|---|
| **Gram$^+$ bacteria** | i15:0 | 0.0175 | 0.2171 | 0.8825 |
| | a15:0 | 0.0242 | 1.7284 | 0.2188 |
| | i16:0 | 0.0237 | 1.6062 | 0.2441 |
| | **i17:0** | **0.0430** | **5.8991** | **0.0119** |
| **Gram$^-$ bacteria** | **cy17:0** | **0.0390** | **5.0135** | **0.0198** |
| | cy19:0 | 0.0239 | 1.6448 | 0.2358 |
| **Unspecified bacteria** | 16:1ω7 | 0.0250 | 1.8939 | 0.1891 |
| **Fungi** | 18:2ω6:9c | 0.0298 | 2.9597 | 0.0792 |
| **Microbial respiration** | BAS | 0.0178 | 0.2972 | 0.8267 |
| | $C_{mic}$ | 0.0179 | 0.3145 | 0.8146 |
| | $qO_2$ | 0.0175 | 0.2325 | 0.8719 |
| **Soil properties** | pH | 0.0320 | 3.4554 | 0.0549 |
| | $NO_3^-$ | 0.0211 | 1.0298 | 0.4170 |
| | $NH_4^+$ | 0.0188 | 0.5116 | 0.6825 |
| | $C_{org}$ | 0.0182 | 0.3726 | 0.7745 |
| | $N_{total}$ | 0.0261 | 2.1450 | 0.1524 |
| | $\delta^{13}C$ | 0.0221 | 1.2510 | 0.3384 |
| | $\delta^{15}N$ | 0.0173 | 0.1733 | 0.9122 |

Bacterial and total PLFA content were not significantly affected by the treatments and averaged 6.67 ± 1.67 and 7.00 ± 0.53 nmol $g^{-1}$dry weight, respectively. The $\delta^{13}C$ values of the fungal marker PLFA 18:2ω6,9 were significantly lower in B+ (-29.93 ± 2.00‰) than in B- rhizotrons (-18.75 ± 3.60‰). Also, weighted $\delta^{13}C$ values of bacterial PLFAs were lower in B+ (-26.28 ± 0.97‰) than in B- rhizotrons (-24.40 ± 0.84‰), whereas in A+ rhizotrons (-24.87 ± 1.01‰) they tended to be higher than in A- rhizotrons (-25.82 ± 0.89‰). In general, ash did not significantly influence $\delta^{13}C$ values of marker PLFA (Table 5).

DFA suggests strong similarity in the composition of PLFAs in BB and BA rhizotrons. Both treatments differed strongly from the AA and the control treatment (Fig. 2). Differences were due to low amounts of gram-negative (i17:0) and gram-positive bacteria (cy17:0), higher fungal biomass and low pH (Table 6, 7). Pyrosequencing of the bacterial community revealed high overlap of bacterial phyla and species with little differences between the treatments (Fig. 3).

**Table 7** | Means ± 1 SE of PLFA markers (nmol $g^{-1}$ dry soil weight) of the microbial community.

| | | **Beech absent (B-)** | | **Beech present (B+)** | |
|---|---|---|---|---|---|
| **PLFA marker** | | **Ash absent (A-)** (Control) | **Ash present (A+)** (Ash) | **Ash absent (A-)** (Beech) | **Ash present (A+)** (Mixture) |
| $Gram^+$ bacteria | i15:0 | 0.9 ± 0.2 | 0.81 ± 0.36 | 1.0 ± 0.2 | 1.59 ± 0.35 |
| | a15:0 | 1.4 ± 0.2 | 1.04 ± 0.34 | 1.4 ± 0.2 | 1.93 ± 0.24 |
| | i16:0 | 0.7 ± 0.0 | 0.66 ± 0.12 | 0.8 ± 0.0 | 0.87 ± 0.06 |
| | i17:0 | 0.6 ± 0.0 | 0.74 ± 0.14 | 0.4 ± 0.0 | 0.70 ± 0.09 |
| $Gram^-$ bacteria | cy17:0 | 0.7 ± 0.1 | 0.77 ± 0.13 | 0.6 ± 0.0 | 0.84 ± 0.16 |
| | cy19:0 | 1.2 ± 0.5 | 1.13 ± 0.46 | 1.1 ± 0.2 | 0.74 ± 0.26 |
| Unspecified | 16:1ω7 | 1.3 ± 0.3 | 0.70 ± 0.35 | 0.8 ± 0.2 | 0.98 ± 0.44 |
| Fungi | 18:2ω6, | 0.2 ± 0.1 | 0.18 ± 0.07 | 0.3 ± 0.0 | 0.53 ± 0.15 |

### 3.4 Gamasid mites

The $^{13}C$ and $^{15}N$ from the added ash litter was incorporated into the soil food web as indicated by the label in the predatory mite *H. aculeifer* (Table 5). The signatures suggest that incorporation of label was most pronounced in mixed rhizotrons (significant interaction between beech and ash) followed by AA, BB and control rhizotrons.

## 4. Discussion

### 4.1 Changes in the microbial community due to rhizodeposition

Lower pH in the rhizosphere of beech likely contributed to favoring soil fungi supporting our hypothesis (1) that beech and ash differentially affect the structure of the microbial community. Acidification of the soil by beech is well known (Holzwarth et al., 2011; Langenbruch et al., 2012), however, commonly it has been ascribed to low concentrations of calcium and magnesium and high concentrations of recalcitrant compounds such as lignin in beech leaf litter (Reich et al., 2005; Hobbie et al., 2006; Hansen et al., 2009). As we excluded leaf litter fall from saplings to the rhizotrons soil surface and uniformly placed high quality ash litter in each of the treatments, the observed differences must have been due to the activity of beech roots. Indeed, in the vicinity of beech roots concentrations of formate and acetate were increased as compared to control rhizotrons in the same experiment, whereas in the vicinity of ash roots only the concentration of acetate increased (Fender et al., 2012). The release of organic acids increases nutrient availability by functioning as reducing agent which is facilitated by low pH (Jones et al., 2004), probably a strategy of beech to make nutrients sequestered in its recalcitrant litter better available. Low pH in the soil, however, predominantly is caused by the release of $H^+$ by roots rather than by dissociation of organic acids (Neumann and Römheld, 1999). Accordingly, $H^+$ concentration was significantly higher in rhizotrons with beech, i.e. the pH was lower. Notably, acidification of the soil by beech roots occurred despite a comparatively lower root biomass in beech than ash rhizotrons. However, SRA and SRL were higher in B+ rhizotrons as compared to A+ rhizotrons. This suggests that the observed modifications were partly due to changes in root physiology rather than root biomass and number of fine root tips (Lehmann, 2003). Differences in the release rates of specific exudates of the two species presumably also contributed to the observed changes.

Bacterial community composition was little affected by tree roots as indicated by analysis of 16S rRNA. However, the ratio of fungal-to-bacterial biomass measured with PLFA analysis increased in B+ rhizotrons and reflected the general pattern of increasing fungal dominance at low pH accounting for differences in soil processes (Aciego Pietri and Brookes, 2008; Rousk et al., 2009). Fungal biomass was measured using 18:2ω6,9 as marker PLFA (Ruess and Chamberlain, 2010; Frostegård et al., 2011) which includes EM and saprotrophic fungi (Kaiser et al., 2010). We suggest the change in fungal biomass only to refer to EM and

saprotrophic fungi, as the PLFA 18:2ω6,9 is only found in very low densities in AM fungi (Olsson and Johansen, 2000) and since the AM colonization rate did not change. Colonization by EM fungi in beech was relatively low (46 ± 6%, pooled data from BB and BA rhizotrons). This corresponds to low colonization rates in other greenhouse and rhizotron experiments (Dučić et al., 2009; Reich et al., 2009; Winkler et al., 2010) when compared to field data (Leuschner et al., 2004; Lang et al., 2011) suggesting that saprotrophic rather than EM fungi increased in beech rhizotrons. Further, lower $\delta^{13}C$ values of PLFA 18:2ω6,9 as compared to fine roots indicate that saprotrophic fungi substantially contributed to changes in the fungal marker PLFA.

Combined data on PLFAs, soil properties and microbial respiration in DFA revealed high similarity of beech and mixed rhizotrons with these differing significantly from ash and control rhizotrons. The fatty acids i17:0 and cy17:0 contributed most to this separation, with slighter contributions by pH and fungal biomass. The fatty acid i17:0 is regarded as marker for gram-positive bacteria whereas cy17:0 characterizes gram-negative bacteria, the former considered to dominate in microorganisms being present in bulk soil whereas the latter in rhizosphere soil processing labile root derived carbon (Söderberg et al., 2004; Paterson et al., 2007). The relative abundance of both was lowest in BB rhizotrons suggesting that both suffered from the presence of beech roots, presumably due to beech increasing the competitive strength of saprotrophic fungi.

### 4.2 Changes in decomposition due to different tree species

Hypothesis (2) assuming that litter decomposition is differentially affected by tree species was supported by our data. Generally, stable isotope values of the litter-soil mixture in MS decreased strongly during incubation by -88% and -81% for $^{13}C$ and $^{15}N$, respectively. Ash litter is known to decompose fast; in the field it disappears entirely after two years (Jacob et al., 2009). High and constant temperatures within the climate chambers (20°C) contributed to fast decomposition of the litter in the rhizotrons (Moore-Kucera and Dick, 2008). Data on higher $qO_2$ (this study) and higher cumulative heterotrophic $CO_2$ production in beech as compared to ash rhizotrons (Fender et al., 2012) suggest an overall higher stimulation of litter decomposition in beech root affected soil, i.e., higher carbon loss due to microbial respiration. High $H^+$ concentrations were shown to limit bacterial growth, while low concentrations limit fungal growth (Rousk et al., 2009). Since bacterial biomass did not decrease we suggest that bacteria were not repressed but their metabolic costs increased reflected in a higher $qO_2$. By

lowering pH beech decreased the efficiency of bacteria to use carbon for biomass production due to increased respiratory losses leading to higher carbon loss from soil. $\delta^{13}C$ values in fungal and bacterial PLFAs were depleted most in B+ rhizotrons suggesting that bacteria and fungi incorporated less litter carbon in presence of beech roots than of ash also indicating a faster turnover of litter carbon. Further, the more depleted $\delta^{13}C$ values in fungi compared to fine roots suggest that fungal carbon originated from soil organic matter, whereas higher $\delta^{13}C$ values in bacteria rather suggest bacteria to depend on root-derived carbon as their signatures resembled that of beech fine roots (Bowling et al., 2008).

Several studies found plant species identity to have stronger effects than plant diversity (De Deyn et al., 2004; Hättenschwiler and Gasser, 2005; Ball et al., 2009), as distinct plant species act as key species (Jacob et al., 2009). The strong effect of beech in this study is mediated by roots whereas ash had no effect suggesting that rhizodeposition in ash is of minor importance. Despite this low rhizosphere changes ash incorporated more litter nitrogen than beech (Lang and Polle, 2011; Schulz et al., 2011); potentially, ash is more effective in exploiting resources from fast decomposing litter such as ash leaves or by virtue of the higher root biomass production of ash in our experiment. Notably, ash saplings incorporated more litter $^{15}N$ than beech saplings supporting the conclusion that the reduced $N_{total}$ in B+ rhizotrons was due to increased SOM decomposition and not due to plant uptake by beech. Notable the uptake of $^{15}N$ declined in mixture with ash. This corresponds to field observations where the N concentrations in ash declined in mixtures with other tree species and their ectomycrrhizal diversity (Lang and Polle, 2011) A higher uptake of N by ash roots was also found in a $^{15}N$ tracer study in the Hainich forest where ash fine roots showed a significantly higher mass-specific uptake of labeled $NH_4^+$ and glycine (but not of $NO_3^-$) than beech roots (A. Jacob, unpubl.).

### 4.3 Channeling of litter-derived carbon into higher trophic levels

Hypothesis (3) assuming that mixing of both tree species beneficially affects the microorganisms thereby stimulating carbon turnover is supported in part by our data. Generally, mixing of tree species increased plant biomass, fine root tips, SRA, SRL and mycorrhizal colonization especially that of beech saplings but did not affect soil chemistry and microorganisms. However, soil chemistry and microbial data are point measures and do not reflect fluxes over the whole period of the experiment. As the plants are sinks for

resources made available over the whole experimental time higher plant growth in mixed rhizotrons suggests that the gross flux of resources was greater in these rhizotrons.

Isotope analyses of food web components are a net measure over the long experimental period. Here, we measured $\delta^{13}C$ and $\delta^{15}N$ being incorporated within the predatory mite *H. aculeifer*. $\delta^{13}C$ and $\delta^{15}N$ values of *H. aculeifer* were significantly increased in mixed rhizotrons suggesting that more litter-derived carbon and nitrogen entered the soil food web. In contrast, in control and beech rhizotrons $\delta^{13}C$ values of *H. aculeifer* resembled those in the field ($\delta^{13}C$: -23.9 ± 0.76‰; $\delta^{15}N$: +2.0 ± 2.11‰; Klarner et al., 2012) suggesting low incorporation of litter-derived carbon (and nitrogen) into the animal food web. However, the turnover of belowground C in unplanted soil, i.e., the control, was numerously shown to be lower compared to planted soil (Kuzyakov, 2010; Bird et al., 2011), i.e. soil with beech trees. Low incorporation of litter resources in BB rhizotrons may point to the fast decomposition of ash litter and to the dominance of root derived resources as basis of the soil animal food web in beech forests as suggested earlier (Pollierer et al., 2007).

## 5. Conclusions

The results suggest that the effect of living roots on litter decomposition, SOM dynamics and energy channels varies with tree species identity. Rhizodeposits have the potential to change soil pH with the potential to affect the metabolic activity of microorganisms. This propagates to higher trophic levels as tree species can impact the amount of litter-derived resource entering the soil food web and on energy channels. Effects of living roots are notoriously understudied and have to be included into studies on soil C dynamics to understand carbon and nutrient cycling as well as soil food web functioning of forests.

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## Figure legends

**Fig. 1** Scheme of double split-root rhizotrons. (a) Front view of mixed species treatments with ash (left) and beech (right) roots interacting in the central compartment. Circles represent manipulation sites (MS) with soil (open circles) or soil-litter mixture (grey circles). The shaded area refers to the surrounding sampling site (SS). Roman numerals indicate soil depths (I-VI). The bold rim in the central compartment from soil depth II to VI represents the sampling area. Black dots along the central dashed line refer to the position of temperature sensors. Dashed lines mark the sampling grid. (b) Side view of the double split-root rhizotron and assembly of MS. Tubes inside MS can be withdrawn to fill them with litter and/or soil. A water flux based cooling system is installed at the back panel. A valve allowed drainage of the rhizotrons.

**Fig. 2** Discriminant function analysis (DFA) of microbial PLFAs, microbial respiration and soil properties in rhizotrons without trees (control), with beech, ash and a mixture of beech and ash. Wilks' Lambda: 0.016480, F (54,33) = 1.85, $P = 0.0296$. Ellipses represent confidence intervals at $P = 0.05$.

**Fig. 3** Discriminat function analysis (DFA) of bacterial phyla based on pyrosequencing of 16S rRNA in rhizotrons without trees (control) and with beech and ash saplings after reducing data to six dimensions by multidimensional scaling (MDS). Wilks' Lambda: 0.499576; $F_{(12,60)} = 2.07$; $P = 0.0325$. Ellipses represent confidence intervals at $P = 0.05$.

**Table 2** Soil characteristics (means ± 1 SE) at the start of the experiment.

| **Soil parameter** | | | |
|---|---|---|---|
| pH | 4.56 | ± | 0.03 |
| CEC [$\mu mol_c\ g^{-1}$ dry weight] | 191.70 | ± | 11.80 |
| Base saturation [%] | 22.90 | ± | 1.30 |
| $N\text{-}NO_3^-$ [$mg\ kg^{-1}$ dry weight] | 4.89 | ± | 0.20 |
| $N\text{-}NH_4^+$ [$mg\ kg^{-1}$ dry weight] | 6.00 | ± | 0.22 |
| $C_{org}$ [$g\ kg^{-1}$ dry weight] | 19.20 | ± | 0.30 |
| $N_{total}$ [$g\ kg^{-1}$ dry weight] | 1.64 | ± | 0.01 |
| C/N ratio [$g\ g^{-1}$] | 11.70 | ± | 0.14 |
| $C_{mic}$ [$\mu g\ C\ g^{-1}$ dry weight] | 382.80 | ± | 14.60 |

CEC: cation exchange capacity; $C_{mic}$: microbial carbon

**Table 2:** Means ± 1 SE and *T*- and *P*-values of plant biomass of beech and ash saplings at the start of the experiment (in g plant$^{-1}$; n=5).

| | Initial Biomass | | | | | |
|---|---|---|---|---|---|---|
| | Beech | | Ash | | | |
| | Means | SE | Means | SE | *T* | *P* |
| **Biomass** | | | | | | |
| Total | 2.04 | ± 0.46 | 2.13 | ± 0.22 | 0.15 | 0.7122 |
| Total aboveground | 1.26 | ± 0.27 | 1.25 | ± 0.15 | 0.01 | 0.9294 |
| Total belowground | 0.78 | ± 0.20 | 0.88 | ± 0.08 | 0.81 | 0.3933 |
| Shoots | 0.88 | ± 0.22 | 0.74 | ± 0.09 | 0.27 | 0.6190 |
| Leaves | 0.38 | ± 0.05 | 0.52 | ± 0.07 | 2.49 | 0.1530 |
| Fine roots | 0.16 | ± 0.05 | 0.41 | ± 0.08 | **6.49** | **0.0343** |
| Coarse roots | 0.64 | ± 0.17 | 0.56 | ± 0.06 | 0.08 | 0.7866 |

**Table 3** Isotopic signatures of the used soil, labeled ash litter and of the soil-litter-mixture in manipulation sites at the start of the experiment and at the end after 422 days of litter incubation (means ± 1 SE).

| | Start | | | End | |
|---|---|---|---|---|---|
| | **Soil** | **Litter** | **Soil-litter mixture** | **Soil-litter mixture** | **Difference* [%]** |
| **$\delta^{13}C$ [‰]** | - 26.20 ± 0.10 | 146.80 ± 0.32 | 69.00 ± 0.60 | -17.44 ± 1.86 | 88.25 |
| **$\delta^{15}N$ [‰]** | 1.60 ± 0.16 | 13139.30 ± 59.10 | 6153.80 ± 0.40 | 577.38 ± 124.88 | 81.23 |
| **C [%]** | 1.92 ± 0.03 | 36.05 ± 0.09 | 5.93 ± 0.05 | 1.94 ± 0.06 | 65.34 |
| **N [%]** | 0.16 ± 0.00 | 1.85 ± 0.01 | 0.40 ± 0.00 | 0.18 ± 0.004 | 54.82 |
| **C/N** | 11.70 ± 0.10 | 19.50 ± 0.10 | 14.90 ± 0.10 | 10.98 ± 0.12 | 15.33 |

* Differences between the signatures from the start and the end of the experiment are displayed for soil samples from manipulation sites overall treatments (n=16) and were related to natural isotopic signatures of ash litter (V. Eißfeller, unpubl. data).

**Table 4** Means ± 1 SE for plant parameters per plant in rhizotrons planted with beech trees (BB), ash trees (AA), beech trees in mixture (BA) and ash trees in mixture (AB) as well as GLM table of contrasts after 475 days.

| | **BB** (pure beech) | **AA** (pure ash) | **BA** (beech in mixture) | **AB** (ash in mixture) | **Pure beech (BB) vs. pure ash (AA)** | | **Pure beech (BB) vs. beech in** | | **Pure ash (AA) vs. ash in** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **S** | **S** | **S** | **S** | ***F*** | ***P*** | ***F*** | ***P*** | ***F*** | ***P*** |
| **Biomass [g dry** | | | | | | | | | | |
| Total | 4.5 ± 0.7 | 12. ± 1.6 | 11. ± 2.0 | 13. ± 2.3 | **8.8** | **0.0** | **6.3** | **0.0** | 0. | 0.9 |
| Total | 1.8 ± 0.3 | 3.3 ± 0.8 | 4.2 ± 1.0 | 3.0 ± 0.4 | 4.2 | 0.0 | 2.9 | 0.1 | 1. | 0.3 |
| Total root | 2.6 ± 0.5 | 8.7 ± 1.0 | 7.0 ± 1.0 | 10. ± 2.1 | **9.5** | **0.0** | **6.9** | **0.0** | 0. | 0.6 |
| Fine roots | 0.7 ± 0.1 | 2.3 ± 0.3 | 1.8 ± 0.2 | 2.2 ± 0.4 | **9.1** | **0.0** | 4.6 | 0.0 | 0. | 0.7 |
| Coarse roots | 1.8 ± 0.3 | 6.3 ± 0.8 | 5.2 ± 0.8 | 8.2 ± 1.9 | **7.9** | **0.0** | **6.5** | **0.0** | 0. | 0.4 |
| **$\delta^{13}C$ [‰] Plant** | | | | | | | | | | |
| Shoot | - ± 0.3 | - ± 0.2 | - ± 0.2 | - ± 0.2 | **5.1** | **0.0** | **7.0** | **0.0** | 2. | 0.1 |
| Leaves | - ± 0.5 | - ± 0.2 | - ± 0.4 | - ± 0.2 | 0.3 | 0.5 | 0.2 | 0.6 | 0. | 0.4 |
| Fine roots | - ± 0.3 | - ± 0.8 | - ± 0.1 | - ± 0.2 | **8.2** | **0.0** | 0.0 | 0.8 | 0. | 0.9 |
| Coarse roots | - ± 0.3 | - ± 0.7 | - ± 0.3 | - ± 0.3 | **12.** | **0.0** | 2.7 | 0.1 | 0. | 0.8 |
| **$\delta^{15}N$ [‰] Plant** | | | | | | | | | | |
| Shoot | 171 ± 30. | 260 ± 66. | 154 ± 18. | 154 ± 26. | 0.8 | 0.3 | 0.0 | 0.8 | 2. | 0.1 |
| Leaves | 192 ± 32. | 316 ± 43. | 166 ± 23. | 228 ± 15. | **5.3** | **0.0** | 0.5 | 0.4 | 1. | 0.1 |
| Fine roots | 209 ± 41. | 396 ± 99. | 148 ± 17. | 214 ± 22. | **4.7** | **0.0** | 1.3 | 0.2 | 4. | 0.0 |
| Coarse roots | 193 ± 27. | 390 ± 78. | 178 ± 12. | 257 ± 19. | **9.3** | **0.0** | 0.1 | 0.7 | 2. | 0.1 |
| **SRA [cm² $g^{-1}$]** | | | | | | | | | | |
| Fine roots | 485 ± 15. | 456 ± 42. | 509 ± 54. | 410 ± 64. | 0.2 | 0.6 | 0.0 | 0.8 | 0. | 0.3 |
| **SRL [cm $g^{-1}$]** | | | | | | | | | | |
| Fine roots | 237 ± 22 | 141 ± 16 | 323 ± 84 | 181 ± 45 | 2.8 | 0.1 | 0.5 | 0.4 | 0. | 0.6 |
| **Fine root tips** | | | | | | | | | | |
| Total number | 162 ± 23 | 229 ± 41 | 307 ± 20 | 354 ± 10 | - | 0.9 | - | **0.0** | 2. | 0.1 |
| **Mycorrhiza** | | | | | | | | | | |
| Colonization | 37. ± 8.5 | 81. ± 5.1 | 54. ± 6.5 | 83. ± 2.8 | **27.** | **0.0** | 3.0 | 0.1 | 0. | 0.8 |

†Note that the different type of mycorrhiza in beech and ash demanded for special counting techniques, thus direct comparisons have to be treated with caution but allow comparison with trees in the mixture.

**Table 5** Means ± 1 SE for soil and microbial parameters and on signatures in gamasid mited as influenced by beech (B) and ash (A) as well as the ANOVA table of *F*- and *P*-values in rhizotrons after 475 days.

| | Beech absent (B-) | | | | Beech present (B+) | | | | Beech (B) | | Ash (A) | | B × A | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ash absent (A-) | | Ash present (A+) | | Ash absent (A-) | | Ash present (A+) | | | | | | | |
| | Me | SE | Me | SE | Me | SE | Me | SE | *F* | *P* | *F* | *P* | *F* | *P* |
| **Soil data** | | | | | | | | | | | | | | |
| pH ($H_2O$) | 4.7 | ± 0. | 4.8 | ± 0.0 | 4.5 | ± 0. | 4.55 | ± 0. | **5.** | **0.0** | 0.1 | 0.7 | 0. | 0.8 |
| N-$NO_3^-$ [mg | 41. | ± 4. | 42. | ± 3.2 | 39. | ± 3. | 35.1 | ± 5. | 1. | 0.3 | 0.0 | 0.8 | 0. | 0.4 |
| N-$NH_4^+$[mg | 2.4 | ± 0. | 1.4 | ± 0.6 | 1.8 | ± 0. | 1.86 | ± 0. | 0. | 0.9 | 0.4 | 0.5 | 0. | 0.5 |
| $C_{org}$ [mg $kg^{-1}$ | 1.8 | ± 0. | 1.9 | ± 0.0 | 1.7 | ± 0. | 1.77 | ± 0. | **15** | **0.0** | 0.0 | 0.7 | 0. | 0.8 |
| $\delta^{13}C$ soil [‰] | - | ± 0. | - | ± 1.3 | - | ± 0. | - | ± 0. | **7.** | **0.0** | 1.7 | 0.2 | 1. | 0.2 |
| $N_{total}$ [mg $kg^{-1}$ | 0.1 | ± 0. | 0.1 | ± 0.0 | 0.1 | ± 0. | 0.16 | ± 0. | **7.** | **0.0** | 0.2 | 0.6 | 0. | 0.9 |
| $\delta^{15}N$ soil [‰] | 212 | ± 55 | 318 | ± 78. | 126 | ± 37 | 127. | ± 20 | **7.** | **0.0** | 0.8 | 0.3 | 0. | 0.5 |
| C/N | 10. | ± 0. | 10. | ± 0.0 | 10. | ± 0. | 10.8 | ± 0. | 0. | 0.4 | 0.9 | 0.3 | 0. | 0.9 |
| **Microbial** | | | | | | | | | | | | | | |
| BAS [µl $O_2$ $h^{-1}$ | 1.1 | ± 0. | 1.1 | ± 0.0 | 1.4 | ± 0. | 1.36 | ± 0. | 4. | 0.0 | 0.0 | 0.7 | 0. | 0.6 |
| $C_{mic}$ [µg C $g^{-1}$] | 150 | ± 13 | 134 | ± 5.9 | 139 | ± 6. | 140. | ± 13 | 0. | 0.8 | 0.4 | 0.5 | 0. | 0.5 |
| $qO_2$ [µl $O_2$ $mg^-$ | 0.0 | ± 0. | 0.0 | ± 0.0 | 0.0 | ± 0. | 0.01 | ± 0. | **9.** | **0.0** | 0.1 | 0.7 | 1. | 0.2 |
| **PLFA [nmol g$^-$** | | | | | | | | | | | | | | |
| Total | 7.2 | ± 1. | 6.0 | ± 1.3 | 6.5 | ± 0. | 8.19 | ± 0. | 0. | 0.4 | 0.0 | 0.9 | 1. | 0.3 |
| Bacteria | 6.9 | ± 1. | 5.8 | ± 1.2 | 6.2 | ± 0. | 7.66 | ± 0. | 0. | 0.4 | 0.0 | 0.9 | 1. | 0.3 |
| Fungi | 0.2 | ± 0. | 0.1 | ± 0.0 | 0.3 | ± 0. | 0.53 | ± 0. | 3. | 0.0 | 0.1 | 0.6 | 1. | 0.2 |
| Fungi/Bacteria | 0.0 | ± 0. | 0.0 | ± 0.0 | 0.0 | ± 0. | 0.07 | ± 0. | **5.** | **0.0** | 0.3 | 0.5 | 0. | 0.3 |
| **PLFA δ $^{13}$C [‰]** | | | | | | | | | | | | | | |
| Total | - | ± 2. | - | ± 2.0 | - | ± 0. | - | ± 2. | 2. | 0.1 | 1.4 | 0.2 | 0. | 0.5 |
| Bacteria | - | ± 1. | - | ± 1.0 | - | ± 0. | - | ± 1. | 2. | 0.1 | 0.4 | 0.4 | 0. | 0.4 |
| Fungi | - | ± 6. | - | ± 4.5 | - | ± 0. | - | ± 4. | **7.** | **0.0** | 0.0 | 0.7 | 0. | 0.6 |
| **Gamasid mites** | | | | | | | | | | | | | | |
| **δ $^{13}$C [‰]** | - | ± 0. | - | ± 0.3 | - | ± 1. | - | ± 0. | **20** | **0.0** | **159** | | **7.** | **0.0** |
| **δ $^{15}$N [‰]** | 130 | ± 23 | 713 | ± 43. | 339 | ± 37 | 112 | ± 26 | **25** | **0.0** | **148** | | **11** | **0.0** |

**Table 6** Summary of input variables of the discriminant function analysis (DFA), i.e. data on PLFA, soil properties and microbial respiration.

| | | Wilks' Lambda | F (3,11) | *P*-level |
|---|---|---|---|---|
| **Gram$^+$ bacteria** | i15:0 | 0.0175 | 0.2171 | 0.8825 |
| | a15:0 | 0.0242 | 1.7284 | 0.2188 |
| | i16:0 | 0.0237 | 1.6062 | 0.2441 |
| | **i17:0** | **0.0430** | **5.8991** | **0.0119** |
| **Gram$^-$ bacteria** | **cy17:0** | **0.0390** | **5.0135** | **0.0198** |
| | cy19:0 | 0.0239 | 1.6448 | 0.2358 |
| **Unspecified bacteria** | 16:1ω7 | 0.0250 | 1.8939 | 0.1891 |
| **Fungi** | 18:2ω6:9c | 0.0298 | 2.9597 | 0.0792 |
| **Microbial respiration** | BAS | 0.0178 | 0.2972 | 0.8267 |
| | $C_{mic}$ | 0.0179 | 0.3145 | 0.8146 |
| | $qO_2$ | 0.0175 | 0.2325 | 0.8719 |
| **Soil properties** | pH | 0.0320 | 3.4554 | 0.0549 |
| | $NO_3^-$ | 0.0211 | 1.0298 | 0.4170 |
| | $NH_4^+$ | 0.0188 | 0.5116 | 0.6825 |
| | $C_{org}$ | 0.0182 | 0.3726 | 0.7745 |
| | $N_{total}$ | 0.0261 | 2.1450 | 0.1524 |
| | $\delta^{13}C$ | 0.0221 | 1.2510 | 0.3384 |
| | $\delta^{15}N$ | 0.0173 | 0.1733 | 0.9122 |

**Table 7** Means ± 1 SE of PLFA markers of the microbial community in rhizotrons after 475 days.

| | | **Beech absent (B+)** | | **Beech present (B-)** | |
|---|---|---|---|---|---|
| | | **Ash absent (A-)** | **Ash present (A+)** | **Ash absent (A-)** | **Ash present (A+)** |
| **PLFA marker** | | (Control) | (Ash) | (Beech) | (Mixture) |
| $Gram^+$ bacteria | i15:0 | 0.92 ± 0.22 | 0.81 ± 0.36 | 1.05 ± 0.21 | 1.59 ± 0.35 |
| | a15:0 | 1.41 ± 0.29 | 1.04 ± 0.34 | 1.40 ± 0.24 | 1.93 ± 0.24 |
| | i16:0 | 0.70 ± 0.08 | 0.66 ± 0.12 | 0.80 ± 0.06 | 0.87 ± 0.06 |
| | i17:0 | 0.62 ± 0.05 | 0.74 ± 0.14 | 0.42 ± 0.04 | 0.70 ± 0.09 |
| $Gram^-$ bacteria | cy17:0 | 0.72 ± 0.16 | 0.77 ± 0.13 | 0.63 ± 0.06 | 0.84 ± 0.16 |
| | cy19:0 | 1.22 ± 0.58 | 1.13 ± 0.46 | 1.13 ± 0.22 | 0.74 ± 0.26 |
| Unspecified | 16:1ω | 1.35 ± 0.37 | 0.70 ± 0.35 | 0.81 ± 0.21 | 0.98 ± 0.44 |
| Fungi | 18:2ω | 0.27 ± 0.16 | 0.18 ± 0.07 | 0.33 ± 0.05 | 0.53 ± 0.15 |

# Appendix 2

Beech carbon productivity as driver of ectomycorrhizal abundance and diversity (2009), Drübert, C., Lang, C., Valtanen, K. & Polle, A. *Plant, Cell and Environment* **32**, 922-1003.

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# Beech carbon productivity as driver of ectomycorrhizal abundance and diversity

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## ABSTRACT

**We tested the hypothesis that carbon productivity of beech (*Fagus sylvatica*) controls ectomycorrhizal colonization, diversity and community structures. Carbon productivity was limited by long-term shading or by girdling. The trees were grown in compost soil to avoid nutrient deficiencies. Despite severe limitation in photosynthesis and biomass production by shading, the concentrations of carbohydrates in roots were unaffected by the light level. Shadeacclimated plants were only 10% and sun-acclimated plants were 74% colonized by ectomycorrhiza. EM diversity was higher on roots with high than at roots with low mycorrhizal colonization. Evenness was unaffected by any treatment. Low mycorrhizal colonization had no negative effects on plant mineral nutrition. In girdled plants mycorrhizal colonization and diversity were retained although $^{14}C$-leaf feeding showed almost complete disruption of carbon transport from leaves to roots. Carbohydrate storage pools in roots decreased upon girdling. Our results show that plant carbon productivity was the reason for and not the result of high ectomycorrhizal diversity. We suggest that ectomycorrhiza can be supplied by two carbon routes: recent photosynthate and stored carbohydrates. Storage pools may be important for ectomycorrhizal survival when photoassimilates were unavailable, probably feeding preferentially less carbon demanding EM species as shifts in community composition were found.**

*Key-words*:beech;diversity;invisibility;nitrogen;productivity; understory ecology.

## INTRODUCTION

Soil microbes are considered as important drivers of plant diversity and productivity in terrestrial ecosystems (van der Heijden, Bardgett & van Straalen 2008). Mycorrhizal fungi play a pivotal role in this respect. In boreal and temperate forests, which are usually nitrogen limited, trees depend on their associated ectomycorrhizal mutualists to supply them with sufficient amounts of nutrients (Read & Perez-Moreno 2003). About 75% of annual phosphorus uptake and up to

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80% of nitrogen are derived from mycorrhizal fungi (Simard, Jones & Durall 2002; Hobbie & Hobbie 2006; van der Heijden *et al.* 2008).

The colonization and diversity of ectomycorrhizal fungi at roots of forests trees is usually high (De Roman,Claveria & De Miguel 2005).The factors controlling this diversity are barely understood but abiotic factors such as soil chemistry or anthropogenic impacts such as increasing nitrogen deposition are important components (Brandrud 1995; Markkola *et al.* 1995; Peter, Francois & Egli 2001; Lilleskov *et al.* 2002). Although it is generally assumed that high diversity will stabilize ecosystem functions and services, the role of ectomycorrhizal communities in this respect is unknown (Johnson *et al.* 2005).Experimental evidence supporting functional roles of mycorrhizal diversity in maintaining plant productivity and diversity mainly stems from studies of arbuscular mycorrhizal fungi in grassland ecosystems, where high diversity of arbuscular mycorrhizal fungi enhanced plant productivity or altered the distribution of nutrients amongst co-existing grassland species (Grime *et al.* 1987;van der Heijden *et al.* 1998;van der Heijden *et al.* 2006;Vogelsang, Reynolds & Bever 2006). However, plants are also expected to affect below-ground microbial communities for example by carbon supply and root exudates,thus, providing feedback loops (Bais *et al.* 2006).

Surprisingly little is known on the question how plant productivity affects ectomycorrhizal fungal associations and their diversity and whether plants can control these communities (Johnson *et al.* 2005). Ectomycorrhizal fungal associations are thought to place a high carbon demand on the host plant and, thus, plant-fungal interactions may not always be positive, especially when carbon resources are limited. In microcosm studies, as much as 20 to 30% of current assimilate was allocated to ectomycorrhizal fungi (Finlay & Söderström 1992; Söderström 2002). EM fungi provide the most important path of plant-derived carbon into soil (Godbold *et al.* 2006), and recent estimates suggest figures of up to 22% of total plant carbon allocation to ectomycorrhiza (Hobbie 2006).Already early tracer studies indicate that photoassimilates are rapidly translocated to

the roots of ectomycorrhizal plants (Melin & Nilsson 1957). When the carbon transport pathway to the roots was interrupted by girdling, soil respiration decreased by 50% in a boreal Scots pine forest (Högberg *et al.* 2001).Separation of heterotrophic, root and hyphal respiration indicated that ectomycorrhizal hyphae contributed 25% to soil $CO_2$ flux (Heinemeyer, Hartley & Evans 2007). These examples underline the carbon costs incurred by ectomycorrhizas. If costs and benefits were directly linked,alterations in carbon supply are expected to directly affect root colonization and community structures. However, when carbon flow to ectomycorrhiza was restricted by defoliation of previous-year needles of Scots pine, no effects on below-ground diversity in term of the number of morphotypes or morphotype diversity index were observed, but the formation of sporocarps decreased (Kuikka *et al.* 2003). Other studies indicated that alterations in carbon availability either by defoliation or by enhancing supply through exposure to elevated $CO_2$ affected fungal community structures (Godbold & Berntson 1997; Saikkonen *et al.* 1999; Parrent, Morris & Vigalys 2006).

In forest ecosystems light is frequently a growth limiting resource. Very little is known on whether light-driven increases in productivity affect the abundance,diversity and community structures of ectomycorrhizas. In experimental studies, colonization with arbuscular mycorrhizal fungi showed a positive relationship with light availability (Pearson, Smith & Smith 1991; Vierheilig *et al.* 2002; Gamage, Singhakumara & Ashton 2004; Gehring 2004). In contrast to this, a pot study with birch and conifers under controlled conditions showed no influence of light on ectomycorrhizal colonization (Dehlin *et al.* 2004). Field studies of birch grown in understory, gaps and clearings suggested that increasing light availability would increase ectomycorrhizal colonization of roots (Cheng, Widden & Messier 2005). However, clear correlations were not obtained because other factors such as soil properties and plant age also varied in this field study and the shade-tolerance of birch as an early succession species is limited.

In Central Europe, European beech (*Fagus sylvatica* L.) dominates the natural forest communities forming monospecific stands in a broad range of environmental conditions (Ellenberg 1996).In the juvenile phase,beech trees are very shade–tolerant, but can also adapt to high irradiation displaying considerable phenotypic and ecophysiological plasticity (Johnson *et al.* 1997; Parelle, Roudaut & Ducrey 2006). European beech, like all Fagaceae, forms only ectomycorrhizal symbioses,which are considered as ecologically obligate for this tree species.

In the present study, we used European beech to address the question whether plant productivity affects ectomycorrhizal colonization and diversity. In our field experimental study, young beech trees were grown densely like natural regeneration forming closed canopies in a nutrient rich soil to avoid nitrogen limitations. Net primary productivity was manipulated by long-term shading to test whether differences in productivity would cause changes in the abundance of fungal partners,and in return affect nutrient allocation to plant tissues. To distinguish between recent and stored assimilate supply on abundance and diversity of ectomycorrhizal fungi, trees were girdled in the middle of the growth season.The disruption of carbon transport was controlled in an accompanying experiment by studying $^{14}C$ below ground allocation. The field study was set up to test the following hypotheses: (1) low plant carbon productivity will lead to changes in the ectomycorrhizal fungi community composition, if the fungi have different carbon demand; (2) the relative abundance of ectomycorrhizal colonization will not change if beech was obligatorily ectomycorrhizal; and (3) alternatively, if ectomycorrhizas were controlled by plant carbon productivity, decreased colonization is expected as a consequence of carbon limitation.

## MATERIALS AND METHODS

### Plants, growth conditions, experimental treatments and harvest

Two-year-old beech trees (*F.sylvatica*,L.,provenance North Germany, seed lot 81009) obtained from a nursery (Forstbaumschule Billen, Bösinghausen, Germany) were planted in March 2003 into compost soil in the area of the Forest Botanical Garden (geographical coordinates: 51°31′48″N, 9°56′39″E; University of Göttingen, Göttingen, Germany). A total of 160 trees were planted at a density of 0.4 ¥ 0.2 m. The area was divided in four plots with plastic barriers.Half the number of trees was shaded with nets (Hermann-Meyer KG,Rellingen,Germany) yielding 65% shading in 2003 and 2004. In 2005 and 2006, a second net was installed decreasing ambient light to about 10% of incident irradiation.

Compost soil with a pH of 7.2 and the following nutrient elements concentrations (mg $g^{-1}$ dry mass): N: 6, P: 1.3, K: 6.8, Ca: 30.4, Mg: 5.7, and S: 1.23 was obtained from Kompostwerk GmbH (Niederorla, Germany). The mean seasonal temperatures (and sum of precipitation) in the growth phase from 1st of April to 30th of September were 15.7 °C (280 mm) in 2003,14.2 °C (404 mm) in 2004,14.5 °C (328 mm) in 2005 and 15.4 °C (446 mm) in 2006. When necessary during dry hot periods, all trees were irrigated with tap water.

On the 30th of June 2006, half of the plants of each light level were girdled at the main stem at half of the distance between the bottom and the first side branches. A 2-cmwide strip of bark was removed around the stem initially retaining a small connecting piece (ca. 5 mm width). Since no effects on photosynthesis were found after 4 weeks of girdling, the connecting bark strap was removed on 7 August. Stem radial diameter was measured once a week at a marked position at the stem basis.

No plant died because of girdling; however, some plants had died between 2003 and 2006. Thus, between 30 and 40 plants were available per treatment. Plant height was determined by measuring the length from the stem basis to the top of the leader shoot.Twenty trees of similar height were selected in each treatment for further analysis.Ten of these plants were harvested 5 weeks (first week of August), and a further 10 plants 10 weeks after girdling (second week of September). Immediately after harvest, aliquots of fresh tissues from five plants per treatment were frozen in liquid nitrogen for further biochemical analysis. Root systems of five plants per treatment and harvest date were

stored at 4 °C for mycorrhizal analysis. All plants were used for biomass determination after separation into leaves, stem and branches, coarse and fine roots. Dry mass was determined after drying for 2 weeks at 40 °C.

## Photosynthesis and chlorophyll fluorescence measurements

Gas exchange was determined with an infrared gas analyser (HCM-1000, Walz, Effeltrich, Germany) at saturating photosynthetically active radiation (PAR) of 800 μmol light quanta $m^{-2}$ $s^{-1}$ at 20 °C, and 65% relative air humidity on five plants per treatment 1 week before harvest in September. Light curves were recorded by increasing the light intensity stepwise from zero to 1200 μmol light quanta $m^{-2}$ $s^{-1}$. Measurements were performed in July on non-girdled, shade- and sun-acclimated plants ($n$ = 5 per treatment).Photosynthesis (PS) curves were fitted by exponential growth equations (software Origin7, OriginLab Corporation, Northampton, MA, USA). PAR was measured approximately twice a week between 10 a.m. and 2 p.m. from July to mid-September at the level of the upper leaves of five plants per treatment.

The quantum yield of photochemistry was determined regularly from July until harvest in September (MINIPAM, Walz, Effeltrich, Germany) at ambient conditions as ($F_m'$ - $F_o$)/$F_m'$ (Maxwell & Johnson 2000) ($n$ = 5 plants per treatment).

## Biochemical methods and element analysis

For pigment analysis, frozen leaves were ground with a pestle and mortar in liquid nitrogen to a fine powder, extracted in 80% acetone and the absorption of the chlorophyll *a*, chlorophyll *b* and carotenoids was determined spectrophotometrically as described by Lichtenthaler & Wellburn (1983).

Soluble carbohydrates were determined enzymatically in extracts of leaf and root tissue yielding glucose, fructose and sucrose concentrations (Beutler 1978). Starch was converted to glucose by amyloglucosidase and measured in glucose units.

Nutrientelementsweremeasuredafterpressuredigestion of dry leaf and root tissues in 65% nitric acid by inductively coupled plasma optical emission spectrometry (Spectro Analytical Instruments, Kleve, Germany) (Heinrichs *et al.* 1986).Carbon and nitrogen were determined in an Elemental Analyzer (Carlo Erba, Rodano, Italy).All analyses were conducted in tissues of five plants per treatment.

## Mycorrhizal analysis

Harvested roots were stored at 4 °C and observed within 2 weeks. They were soaked in water and washed gently to remove the adhering soil. Five fine root samples per plant with 200 to 300 root tips each were observed using a dissecting microscope (Stemi SV 11, Zeiss, Jena, Germany).

Root tips were counted and recorded as vital mycorrhizal, vital non-mycorrhizal and dead root tips. To control these classifications, selected root tips were embedded (Gafur *et al.* 2004) and anatomically inspected (data not shown). Relative EM colonization was calculated as EM/(EM + vital non mycorrhizal root tips). Mycorrhizal root tips were divided into morphotypes according to a simplified system of Agerer (1987–2006) based on morphological characters like colour of the mantle, branching and appearance and properties of emanating hyphae and rhizomorphs. All morphotypes were documented with digital photos (Coolpix 4500, Nikon, Düsseldorf, Germany).

To identify EM species DNA was extracted from aliquots of the morphotypes, which had been stored at -80 °C. Frozen EM root tips were ground in a mill (Typ MM 2, Retsch, Haan, Germany) and extracted using the DNeasy® Plant Mini Kit 50 (Qiagen, Hilden, Germany) following the instruction of the manufacturer. The ITS (internal transcribed spacer) region was amplified by PCR using the primers ITS 1 (5′- TCC GTA GGT GAA CCT GCG G-3′) and ITS 4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White *et al.* 1990). PCR was performed according to Landeweert *et al.* (2005). PCR products were cloned in the pGEM®-T System I vector (Promega, Madison, WI, USA) and transformed into electrocompetent *E. coli* cells (TOP TEN, Invitrogen, Carlsbad, CA, USA). Positive clones were sequenced (ABI Prism 3100 Genetic Analyser, 36 cm capillary, Matrix Pop 6, Applied Biosystems Foster City, CA, USA). The sequences were compared with those present in UNITE database (Kõljalg *et al.* 2005, http://unite.ut. ee/) and NCBI database (http://www.ncbi.nlm.nih.gov/). UNITE is a databank for ectomycorrhizal fungi, which requires deposition of specimen and therefore is usually more reliable than NCBI,which is a general database for all kinds of sequences.

## $^{14}C$ glucose below ground allocation

In an independent experiment, one year old beech plants grown in pots in a greenhouse,were transferred to a growth cabinet and maintained under 16 h light (200 μmol photosynthetic active radiation)/8 h darkness, 20 °C and 60 to 70% relative air humidity. Six plants were girdled 3 cm above soil level by removing an about 1-cm-broad strip of bark. Further six plants remained intact.After 1 h the third fully expanded top leaf was rubbed with silicium carbid (‘Carborund’, ESK-SIC GmbH, Frechen, Germany) and immediately supplied with 50 ml 5 mCi glucose (Moravek Biochemicals, Brea, CA, USA). The plants were watered regularly and harvested after 3 and 6 d. The plants were separated into the following fractions: coarse roots, fine roots, a 1-cm-long stem piece directly below the girdle (separated in bark and wood), a 1-cm-long stem pieces directly above the girdle (separated in wood and bark) and residual materials.The plant materials were dried for 24 h at 70 °C, and the exact mass of each plant fraction was determined.The samples were combusted in a biological oxidizer (Ox500, Zinsser, Frankfurt, Germany) using the carbon-14 standard for sample oxidizers from Amersham International (Plc., Amersham, UK) as the standard. Resulting $^{14}CO_2$ was trapped in a liquid scintillation cocktail (Zinsser Analysis Oxysolve TC-400, Zinsser Analytic, Frankfurt, Germany). The radioactivity was measured in a liquid

scintillation analyser (Tri-Carb 2800 TR, PerkinElmer Life Sciences, Wellesley, MA, USA). Total radioactivity was determined in each of the fractions and related to the applied amount of radioactivity: tissue label (%) = (total activity in a plant fraction) * 100/(total applied radioactivity).

## Data analysis

The following diversity indices were calculated: species richness $H(\max) = \ln(\text{number of all species})$, ShannonWiener index, $H' = -\Sigma p_i \ln p_i$, where $p$ is the probability of the species $i$, and Evenness = $H'/H(\max)$ (Shannon & Weaver 1949). The sampling unit 'plant' served as the basis for calculating these indices, if not indicated differently.

Statistical analysis was performed using Statgraphics Plus 3.0 (StatPoint, Inc., St Louis, MO, USA). The experiment had a randomized block design with the factors light and girdling. Data in figures and table are indicated are as means ( 1 SE) when assumptions for normality were met (Shapiro-Wilks Test W, $P <$ 0.05) and were compared by anova and a multiple range test (LSD). If assumptions of normality were not met, data were log transformed before anova. Means were considered to be significantly different from each other, if $P$ 0.05. Significant differences are indicated in tables and figures by different letters. Regression curves were plotted with the programme Origin 7G (Origin Lab, Corporation, Northhampton, MA, USA).

# RESULTS

## Light limitations affect growth but not carbon storage in roots

To limit carbon availability, beech trees were grown for four seasons under limiting light. Maximum rates of lightsaturated photosynthesis were about 2 and 9 mmol $CO_2$ $m^{-2}$ $s^{-1}$ in shade and sun acclimated plants, respectively, in the fourth season (Table 1). Chlorophyll concentrations showed typical adaptations to shade and sun, respectively (Table 1). Under ambient light net photosynthesis (NP) rates were estimated on the basis of light response curves of sun-acclimated plants [NP = 6.59 + (-8.12 e $^{(PAR/-109.75)}$), $r^2$ = 0.9856] and shade-acclimated plants [NP = 2.42 + (-3.11 e $^{(PAR/-56.20)}$), $r^2$ = 0.9848]. Sun-acclimated plants maintained photosynthetic activities close to the maximum, whereas shade-acclimated plants operated just above the light compensation point of 13 mmol light quanta $m^{-2}$ $s^{-1}$ (Table 1). The photosynthetic limitation resulted in significantly diminished growth and biomass production in shade-grown compared with sun-grown trees but not in differences in biomass allocation between above and below ground plant tissues as indicated by similar root-to-shoot ratios of sun- and shade-acclimated plants (Table 1).

Soluble carbohydrate concentrations calculated as the sum of glucose, fructose and sucrose of sun-acclimated leaves were higher than those of shade-acclimated leaves, whereas the foliar starch concentrations were unaffected (Fig. 1a,b). Soluble carbohydrates of coarse and fine roots as well as starch concentrations of fine roots of sunacclimated plants were similar to those of shade-acclimated plants (Fig. 1c,e,f).The starch concentrations in coarse roots of shade-acclimated plants were even higher than those of sun-acclimated plants (Fig. 1d). This indicates that

**Table 1.** Photosynthesis, growth, biomass, pigments and carbohydrates in beech (*Fagus sylvatica*) grown in full sun or with limited light

| | Sun control | Sun girdled | Shade control | Shade girdled |
|---|---|---|---|---|
| PAR (mmol $m^{-2}$ $s^{-1}$)[a] | 980 123b | 990 128b | 20 3a | 19 2a |
| Photosynthesis (estimated) | 6.05 0.28b | 6.14 0.23b | 0.21 0.07a | 0.22 0.11a |
| Photosynthesis (max) | 8.85 1.49b | 9.02 0.39b | 2.21 0.28a | 2.11 0.49a |
| Chl ($a$ + $b$) (mmol $g^{-1}$ FW) | 2061 312a | 1906 105a | 4025 332b | 3729 266b |
| Leader shoot height[a] (m) | 0.88 0.34b | 0.94 0.38b | 0.61 0.20a | 0.56 0.16a |
| Stem increment[a] (‰ per week) | 15.8 5.3b | 12.0 5.9b | 8.4 4.9a | 3.8 3.1a |
| Biomass[a] (g) | 664 131b | 680 146b | 34 8a | 26 2a |
| Root/shoot | 0.55 0.06b | 0.35 0.03a | 0.59 0.03b | 0.49 0.02b |
| NS carbon (mg whole $root^{-1}$) | 3901 157c | 2850 275b | 309 21a | 143 19a |

[a]Data were log-transformed for statistical analysis.

NS, non-structural.

Estimated photosynthesis (mmol $CO_2$ $m^{-2}$ $s^{-1}$) was calculated on the basis of the equations for the light response curves (see text) and repeated light measurements (PAR*) from July to September. Maximum photosynthesis was determined in September at saturating photosynthetically active radiation of 800 mmol quanta $m^{-2}$ $s^{-1}$. Non-structural carbohydrates were determined as the sum of starch,glucose,fructose and sucrose in fine and coarse roots ($n$ = 5, 1 SE). Different letters in rows indicate significant differences at $P$ 0.05.

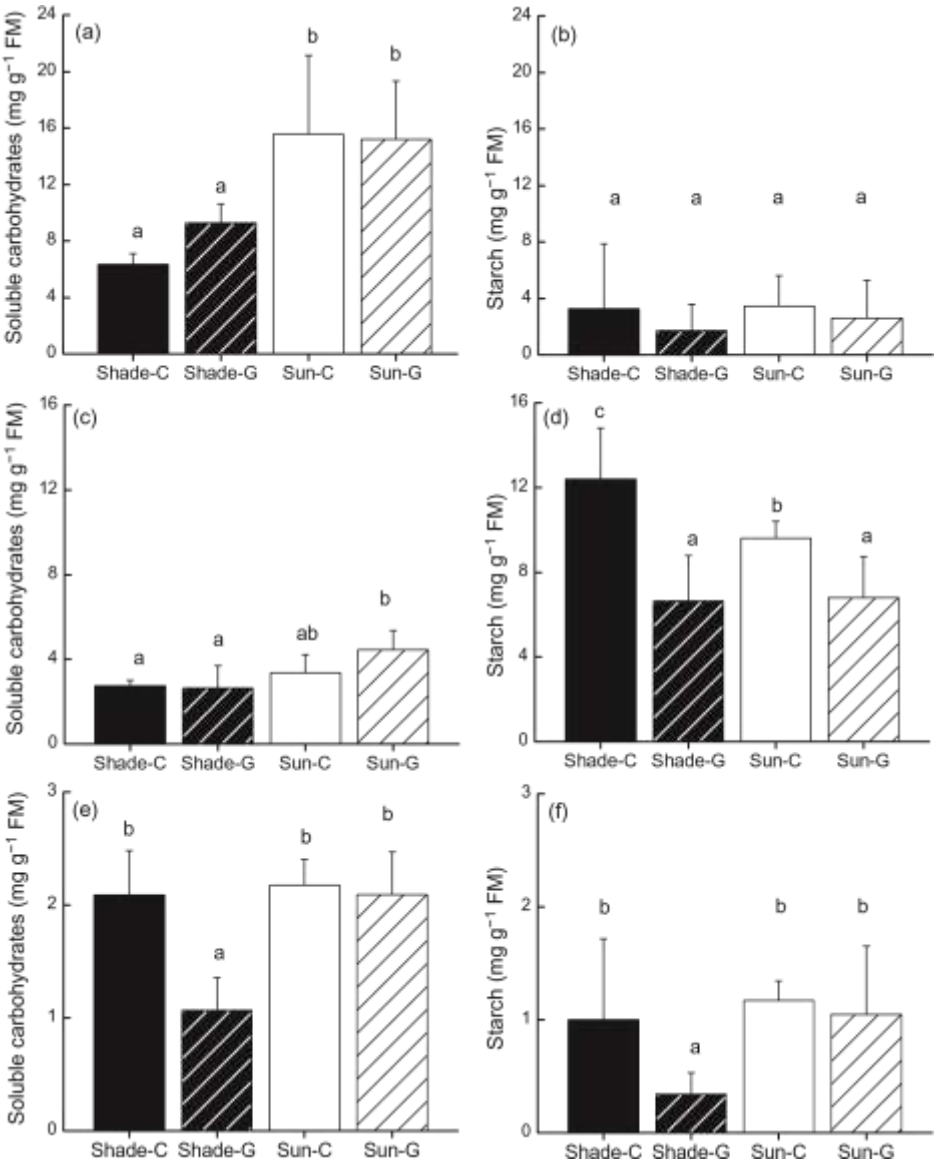


**Figure 1.** Soluble carbohydrates and starch in leaves (a, b), coarse roots (c, d) and fine roots (e, f) of beech (*Fagus sylvatica*) grown in full sun or with limited light. Plants were grown under shading nets with about 10% of incident radiation (black bars) or in full sun (white bars) for 4 years. Half of the plants of each light level were girdled (hatched bars). Soluble carbohydrates were determined as the sum of glucose, fructose and sucrose in September. Data indicate means ($n$ = 5, 1 SE). Different letters in rows indicate significant differences at $P$ 0.05.

despite severe light limitation of photosynthesis, below ground storage pools of carbon were not depleted.

## Girdling has no immediate implications for photosynthesis but leads to depletion in root carbon storage pools

To limit flux of recently fixed carbon to the roots,a subset of trees from each light regime was girdled in the mid of the growth season, initially partly and after five weeks completely. Girdling had no negative effect on quantum yield of photochemistry (not shown), $CO_2$ assimilation or gross biomass but caused a loss in root relative to shoot biomass in sun-acclimated plants (Table 1). Girdling did not affect carbohydrate concentrations in the leaves (Fig. 1a,b).

Since we had expected accumulation of carbohydrates and feed-back inhibition of photosynthesis in response to girdling, we wondered whether the girdle could be circumvented by radial transport of carbohydrates into the xylem ray parenchyma cells, transport in radial and axial ray cells with possible reallocation into the bark below the girdle.To investigate this possibility, a radioactive labelling experiment was conducted under controlled conditions.A mature top leaf was fed with $^{14}C$ glucose and the relative distribution of the label above and below the girdle and

in the root system was analysed after 3 and 7 d. After these exposure

**Table 2.** Carbon transport to roots after girdling

| Treatment | Chase time (d) | Wood (a) (%) | Bark (a) (%) | Wood (b) (%) | Bark (b) (%) | Coarse roots (%) | Fine roots (%) | Through flow (%)[a] |
|---|---|---|---|---|---|---|---|---|
| Control | 3 | 0.13 0.05 | 0.27 0.07 | 0.17 0.03 | 0.35 0.08 | 13.85 2.98 | 4.33 1.07 | 0.121 |
| Girdle | 3 | 0.70 0.22 | 1.09 0.12 | 0.06 0.02 | 0.04 0.01 | 0.00 0.00 | 0.05 0.02 | 0.033 |
| Control | 7 | 0.06 0.01 | 0.17 0.07 | 0.05 0.01 | 0.18 0.08 | 10.00 3.78 | 4.76 1.21 | 0.128 |
| Girdle | 7 | 0.99 0.51 | 1.86 0.64 | 0.07 0.01 | 0.05 0.02 | 0.01 0.00 | 0.04 0.01 | 0.000 |
| $P_{(girdle)}$ | 0.026 | 0.005 | 0.069 | 0.006 | 0.001 | 0.001 | nd | |
| $P_{(time)}$ | 0.693 | 0.337 | 0.026 | 0.218 | 0.447 | 0.800 | nd | |
| Interaction | 0.525 | 0.213 | 0.013 | 0.167 | 0.446 | 0.792 | nd | |

[a]Radioactivity in through flow was determined a pooled sample.

Data indicate fraction of radioactivity (%) recovered in wood, bark, coarse and fine roots of young beech (*Fagus sylvativa*) trees after $^{14}C$ glucose feeding via a leaf. Six trees were girdled 1 h before application of $^{14}C$ glucose to the leaf and six trees were kept as controls. Total radioactivity applied to the leaf was set as 100%. Trees were maintained in a growth cabinet during the labelling and chase phase and harvested 3 and 7 d after labelling ($n$ = 3 per time point and treatment. (a) refers to wood and bark directly above and (b) below the girdle. Controls were harvested at the corresponding positions.

times, coarse and fine roots of non-girdled controls showed a strong accumulation of the applied label, whereas in the root system of girdled trees only traces were detected (Table 2).A sizable fraction of the label was also present in the throughflow of the irrigation water of non-girdled trees, whereas only traces were present in the irrigation water of girdled trees (Table 2). Along the transport path, girdled trees showed an accumulation of the label in xylem and bark above but a strong decrease below the girdle (Table 2). In control trees, no gradient was observed at corresponding positions of the stem.This shows that although girdling does not interrupt belowground carbon allocation completely, the fraction of carbohydrates reaching the belowground compartment was only marginal accounting for 0.01% and 0.25% in coarse and fine roots compared with non-girdled controls.

In the field experiment, the concentration of nonstructural carbon in roots, especially that of starch in coarse roots of shaded plants, decreased as a consequence of girdling (Fig. 1d). As the replenishment of carbon resource in the girdled trees was negligible, carbon consumption rates of the roots can be assessed. Using biomass data for fine and coarse roots, the total pool sizes of the metabolically active carbon fraction (sum of glucose,fructose,sucrose and starch) were calculated (Table 1). Within 10 weeks, girdled sun- and shade-grown beech trees lost 27% and 54%, respectively, of the total carbohydrates compared with nongirdled controls.

## Light limitation and girdling have different impact on mycorrhizal abundance and diversity

Fine roots of beech trees grown under full sun light showed about twice higher fractions of dead root tips than shadeacclimated trees (Fig. 2a). The vital root tips of shadeacclimated trees displayed very low colonization with ectomycorrhizal fungi in comparison with trees grown in full sun light (Fig. 2b). Girdling had no influence on ectomycorrhizal abundance but resulted in a trend towards higher root mortality in roots of shade-acclimated trees (Fig. 2a,b).The maintenance of ectomycorrhizal abundance and proportion of colonization was unexpected and suggested that the observed decreases in root carbohydrate storage were not only utilized for self support of root structures but also to feed mycorrhizas.

To find out whether differences in growth and plant biomass production as well as girdling affected the composition of the fungal communities, morphotyping was combined with sequencing of the ITS region to identify fungi and to obtain quantitative data on root colonization. We have been able to obtain sequence information for 11 out of initially 20 different fungal morphotypes. The 11 sequences belonged to 7 different species yielding a total number of 16 putatively different fungal species forming ectomycorrhizas with the beech trees in this experiment (Supplementary files 1A & B). We can not exclude that these numbers overestimated species richness because we have not been able to obtain PCR products for all morphotypes. However, the non-sequenced morphotypes were dissimilar (Supplementary file 1B). Based on these species numbers, total species richness at roots of shade- and sunacclimated beech trees was 3 and 14, respectively, and not affected by girdling (Supplementary file 1C). Only one species, an uncultured ECM (MT1), occurred at root tips of all treatments.

Shannon-Wiener indices ($H'$) were calculated for each root sample (mean root sample = 988 57 vital root tips, $n$ = 5 per experimental variable).$H'$ was significantly higher for ectomycorrhizas at roots of sun-acclimated than for those of shade-acclimated trees (Fig. 2c). Girdling had no significant influence on $H'$ (Fig. 2c). Overall, these results show that if there were more root tips amenable for mycorrhizal colonization, they were colonized by more different fungal species and not by more fungi of the same species.

To find out whether changes in community composition observed at roots of light-limited and sun-exposed or girdled

and non-girdled trees affected dominance structures, we calculated evenness. There were no significant differences between the treatments. Mean Evenness across all treatments was 0.71 0.02. We conclude that beech trees showed a surprisingly stable pattern of dominance structures of their ectomycorrhizal communities, although the colonization of the roots tips varied strongly (Fig. 2) and the species composition changed with light level and girdling (Supplementary files).

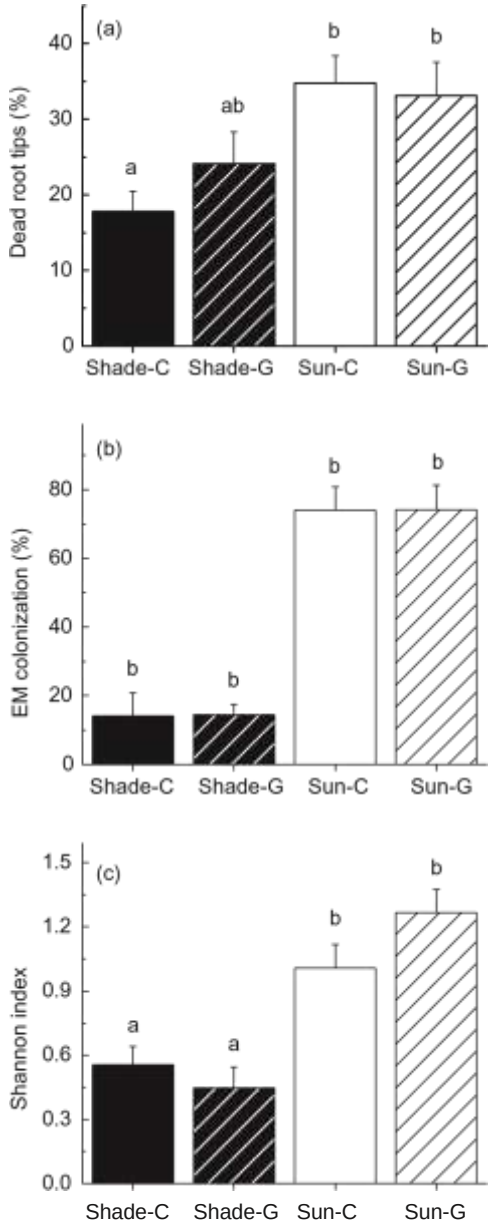


**Figure 2.** Proportion of dead root tips (a), mycorrhizal colonization of vital roots tips (b) and Shannon-Wiener index *H*′ of mycorrhizal roots of beech (*Fagus sylvatica*) grown in full sun or with limited light. Plants were grown under shading nets with about 10% of incident radiation (black bars) or in full sun (white bars) for 4 years. Half of the plants of each light level were girdled (hatched bars). Measurements were taken in August and September. As no significant differences were found between data, means of both sampling dates are shown ($n$ = 10, 1 SE). Different letters in rows indicate significant differences at $P$ 0.05.

## Mycorrhizal abundance and diversity in relation to beech productivity and nutrition

As low rates of mycorrhizal colonization can lead to insufficient nutrient uptake, which in turn may affect growth, nutrient element concentrations were determined in fine roots and leaves (Table 3). The nitrogen concentrations of leaves and roots of the shade-grown plants were higher than those of sun-exposed plants (Table 3). The concentrations of other nutrients were more variable than those of nitrogen. Roots of girdled, sun-exposed trees contained more P than roots of girdled,shade-exposed trees (Table 3). In roots of non-girdled trees, differences between the harvest in August and the harvest in September were observed, but there were no effects of shading (Table 3). Other nutrients were usually higher or unaffected in shadecompared with sun-exposed plants (Table 3). We conclude that the productivity of shade-exposed plants was only limited by light and not by nutrients.Therefore,mycorrhizal colonization and diversity of shaded plants were dependent on plant productivity and not *vice versa*.

We plotted biomass as independent and diversity *H*′ as dependent variable to investigate the relationship between these parameters (Fig. 3). The best fit was obtained for an exponential function (Fig. 3). A similar relationship was obtained for biomass and mycorrhizal colonization rates (Fig. 3) because *H*′ was increased in samples with high mycorrhizal abundance (Fig. 2b,c).

# DISCUSSION

## Beech productivity governs ectomycorrhiza abundance and diversity

Understanding the links between aboveground and belowground communities is one of the challenging tasks of ecology (Bardgett *et al.* 2005). In forest ecosystems, analysis of the relationship between beech productivity and diversity of the associated root fungi is hampered by soil patchiness, nutrient availability, tree age and other variables. To test the hypothesis that plant productivity was the reason for and not the result of high ectomycorrhizal diversity, our study was conducted under conditions where these factors were excluded. A draw-back of such experimental conditions is that the fungal community and colonization found at the roots of beech were not typical of a forest situation. Beech roots are usually almost completely (99%) colonized by a highly diverse ectomycorrhizal flora (Taylor, Martin & Read 2000; Rumberger *et al.* 2004; Buée, Vairelles & Garbaye 2005; Grebenc & Kraigher 2007). Repeated samplings across different seasons revealed up to 90 different morphotypes on beech roots in an undisturbed oldgrowth forest (Pena *et al.*, unpublished results). But species numbers depend on the sampling scheme (Taylor 2002).For example, beech roots sampled once along a north–south transect at sites differing in N pollution showed between 11 and 20 different morphotypes, which is within the range of morphotypes found in our study.

Similar figures were also reported for beech grown on relatively sandy soil

**Table 3.** Nutrient element concentrations (mg $g^{-1}$ dry mass) in leaves and roots of beech (*Fagus sylvatica*) grown in full sun or shade

| | | 1st harvest | | 2nd harvest | | 2nd harvest | |
|---|---|---|---|---|---|---|---|
| Tissue | Element | Shade control | Sun control | Shade control | Sun control | Shade girdled | Sun girdled |
| Leaf | C | 458.3 9.6c | 473.6 4.1e | 433.7 2.0a | 464.5 2.9cd | 445.1 2.7b | 465.7 5.4d |
| Leaf | N | 26.20 0.64cd | 21.69 2.03a | 27.98 2.71d | 23.08 2.38ab | 25.10 2.10bc | 20.93 1.51a |
| Leaf | P | 0.95 0.29a | 1.21 0.07ab | 1.51 0.32c | 1.35 0.18bc | 1.37 0.07bc | 1.24 0.19bc |
| Leaf | K | 8.09 3.75b | 4.51 0.51a | 15.39 1.89d | 6.46 1.09ab | 12.29 2.53c | 4.95 0.69a |
| Leaf | S | 1.21 0.42a | 1.24 0.08a | 1.79 0.18c | 1.52 0.11bc | 1.65 0.17c | 1.27 0.180ab |
| Leaf | Ca | 8.82 3.91a | 10.19 2.41a | 15.21 2.23b | 11.16 2.96a | 10.01 1.0a | 10.43 1.66a |
| Leaf | Mg | 2.17 0.78a | 2.51 0.29ab | 3.05 0.67b | 2.32 0.32a | 3.01 0.21b | 2.16 0.44a |
| Leaf | Mn | 0.02 0.01ab | 0.02 0.01bc | 0.03 0.01d | 0.02 0.01bc | 0.03 0.01cd | 0.01 0.01a |
| Leaf | Fe | 0.44 0.23bc | 0.19 0.09a | 0.81 0.27d | 0.31 0.09ab | 0.56 0.19c | 0.18 0.08a |
| Root | C | 465.5 5.4bc | 454.0 13.4a | 468.2 5.9c | 455.3 12.8ab | 475.2 3.4c | 465.6 1.6bc |
| Root | N | 16.97 2.28b | 11.18 1.56a | 17.13 1.73b | 11.83 0.52a | 16.27 4.33b | 11.39 1.16a |
| Root | P | 1.16 0.57a | 1.62 0.66ab | 1.46 0.67ab | 1.32 0.29a | 1.25 0.39a | 2.17 0.93b |
| Root | K | 3.93 1.75a | 3.78 0.97a | 5.19 1.38a | 4.13 0.27a | 5.00 1.61a | 4.18 0.63a |
| Root | S | 1.59 0.65c | 1.07 0.34ab | 1.32 0.13bc | 1.08 0.19ab | 1.07 0.11ab | 0.97 0.19a |
| Root | Ca | 13.42 5.48b | 9.59 2.91a | 9.53 1.26a | 9.26 2.39a | 9.23 0.81a | 8.75 2.11a |
| Root | Mg | 1.86 0.78a | 1.98 0.43a | 1.81 0.27a | 1.89 0.36a | 1.73 0.38a | 1.88 0.34a |
| Root | Mn | 0.07 0.03ab | 0.07 0.02b | 0.05 0.01ab | 0.05 0.02ab | 0.05 0.02a | 0.05 0.01a |
| Root | Fe | 1.45 0.67abc | 1.85 0.51c | 1.01 0.24a | 1.68 0.83bc | 1.05 0.46ab | 1.14 0.31ab |

Data indicate means ($n$ = 5, SD) of harvest 1 (first week in August) and harvest 2 (second week in September). Different letters in rows indicate significant differences at $P$ 0.05.

(Rumberger *et al.* 2004).We can not exclude that the nutrient rich compost soil, which we used to avoid nutrient limitations affected diversity and root colonization. For example, the unusually high number and abundance of *Tuber* species might have been caused by the relatively high soil pH (Pruett, Bruhn & Mihail 2008). There is evidence that ectomycorrhizal species richness is suppressed by high nitrogen availability (Jonsson, Anders & Tor-Erik 2000; Peter *et al.* 2001; Lilleskov *et al.* 2002; Carfrae *et al.* 2006; Parrent *et al.* 2006; Avis, Mueller & Lussenhop 2008), although the development of species rich nitrophilic communities has also been observed (Kranabetter, Durall &

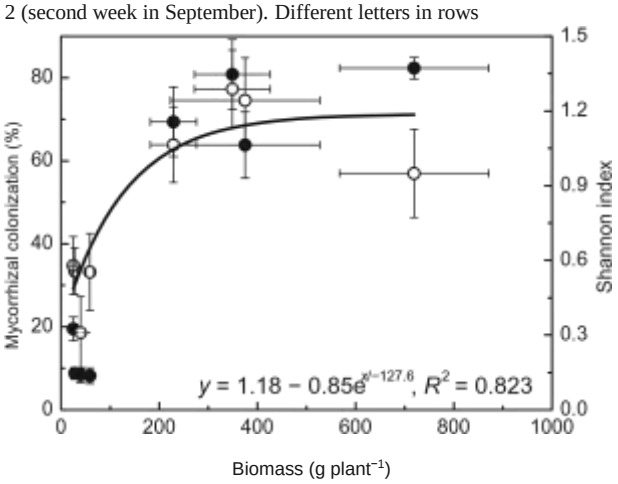


**Figure 3.** Relationship of diversity (white circles) of fungal taxa or proportion of ectomycorrhizal roots colonization (black circles) with biomass of beech ($n$ = 5, 1 SE). Diversity was fitted by exponential increase (line).

MacKenzie 2008). Despite these uncertainties, our study clearly demonstrates that ectomycorrhizal colonization of roots was strongly related to beech net primary productivity and that increasing colonization was accompanied with increasing diversity of the fungal community at root tips.

In our study, shade grown beech trees showed acclimation of photosynthetic parameters and productivity similar to those in

previous studies (Tognetti *et al.* 1998; Lichtenthaler *et al.* 2000).The strong dependence of mycorrhizal colonization on host productivity may shed further light on the significance of below-ground hyphal networks connecting trees with each other in forest ecosystems. Our data suggest that strongly light-limited understory plants may not be able to invest sufficient own carbon into mycorrhizas, whose functions may, however, be essential to satisfy their nitrogen requirement in nutrient-limited environments. In fact, oak seedlings connected with congeneric trees by mycorrhizal nets contained increased foliar nitrogen concentrations compared with unconnected seedlings (Dickie, Koide & Steiner 2002). In understory, the connection of carbonlimited trees with a hyphal web subsidized by light-exposed, mature trees might be ecologically beneficial and grant their survival, even if the actual carbon exchange between the trees is marginal (Robinson & Fitter 1999).

## Functional differences of recent photosynthate and stored carbohydrates for ectomycorrhizas

The observed relationships between mycorrhizal colonization, species richness, and diversity might have been expected according to ecological concepts relating resistance of invasibility of new species to resource limitations (Levine & D'Antonio 1999). In such a system higher

photoassimilate production would increase fine root productivity and, thus, increase the ability of novel EM taxa to invade (Davis, Grime & Thompson 2000). In the wider context of our data, this explanation is, however, too simplistic. First, we always analysed the same number of vital root tips;the high proportion of non-mycorrhizal root tips in light-limited plants indicates that'available' root tip per se is an insufficient indicator to predict ectomycorrhizal diversity. Second, the carbohydrate concentrations in the root systems of sun-acclimated trees were similar to those of light-limited trees.Therefore, root carbohydrate concentrations can also be excluded as the direct cause of increased proportion of root colonization and increased diversity of ectomycorrhizal fungi. It is possible that carbon exudation is required to attract and enable the installation of ectomycorrhiza,and that the interaction is further regulated by hormonal signals (Mathesius 2003; Morgan, Bending & White 2005).The links between such plant-born signals and diversity of EM fungi will have to be addressed in future studies.

As ectomycorrhizal symbiosis involves reciprocal transfer of carbon and nutrients between plant and fungi, feedbacks of low EM colonization on plant nutrition might have been expected. The foliar N concentrations in beech of our study were in the range of those found in field studies across Europe (Bauer, Schulze & Mund 1997; Duquesnay *et al.* 2000). Increased foliar N concentrations in shadeacclimated plants reflect higher investment into photosynthetic structures and excluded negative effects of low EM colonization on nutrient supply to these plants. The sunexposed plants with high biomass production contained lower N concentrations in their tissues, but whole-plant N uptake was strongly increased because of the significant increase in biomass production. Among the abundant EM taxa colonizing roots of sun-exposed trees, two species, an uncultured EM and *Tuber maculatum*, were characterized by emanating hyphae which may extend the soil volume used for nutrient foraging (Agerer 2001). Such EM associations may be important to satisfy higher N demand of sunexposed plants. Furthermore, the maintenance of such species may be too expensive for carbon-limited beech because long distance exploration types of EM fungi, e.g. *Suillus* sp., have higher carbon demand than species with a short radius of soil exploration or species that may acquire carbon by saprotrophic mechanisms, e.g. *Piloderma* sp. (Heinosala, Hurme & Sen 2004; Saravesi *et al.* 2008). Overall, our results show that mycorrhizal colonization is under host control, and that host carbon productivity is a pre-condition for ectomycorrhizal diversity.

This conclusion was also supported by the results of girdling experiments. Ectomycorrhizal fungi are considered important sinks for carbon, which are rapidly supplied with carbohydrates from current photosynthetic activities (Melin & Nilsson 1957; Heinosala *et al.* 2004). In pulsechase feeding experiments with Scots pine, maximal C uptake in the extramatrical mycelium occurred within 3 d (Leake *et al.* 2001). One might, therefore, expect that disruption of this carbon supply would cause a relatively rapid loss in ectomycorrhizas. However, this was neither observed in the present relatively short-term study nor in other girdling experiments with mature beech in forest ecosystems lasting more than 1 year (Pena *et al.*, unpublished results). Similarly, Kuikka *et al.* (2003) and Högberg *et al.* (2001), who restricted carbon flux to roots of Scots pine by defoliation or girdling, respectively, found no rapid loss in ectomycorrhizal colonization proportion or loss in diversity. Since beech – unlike conifers – may have the possibility to circumvent the girdle by carbon transport through axial parenchyma cells, we have also demonstrated that girdling was very efficient in disrupting basipetal carbon transfer. Therefore, our data document that the consumption of carbohydrate reserves must have enabled the maintenance and survival of ectomycorrhizal as well as non-mycorrhizal roots. This was supported by the observation that increases in necromass in response to girdling were not significant. The higher fraction of dead root tips in sun- compared with shade-exposed trees may be caused by slower decomposition of ectomycorrhizal than of non-mycorrhizal roots (Langley, Chapman & Hungate 2006).

In our study, the dominance structures of EM were surprisingly stable since Evenness did not change across all treatments. However, there were small changes in the community composition on roots of girdled trees compared with those of non-girdled trees at the two different sampling dates. At first glance, this was surprising since one might assume that the same host species in same soil might have the same preferences for ectomycorrhizal partners. However,it has also been shown that the carbon demand of fungal taxa is different and that plants allocate more carbon to more beneficial mutualists (Rosling, Landahl & Finlay 2004;Bever *et al.* 2009).Therefore,the community pattern is expected to change, if fungi with lower carbon demand would be favoured or would be more competitive when the supply with recent photoassimilates stopped.We noted that the abundance of a common fungal taxon (MT13) of shadeacclimated trees, which forms only small EM structures with a smooth mantle, increased consistently in girdled sun-acclimated trees. However, since only one taxon was involved, the overall changes in community structures were small.

Our study suggests that carbon stored in the root systems is normally not used by the fungal partners, but can apparently be shunted to support ectomycorrhizal structures if supply with current assimilates is restricted. How this sink regulation functions is an interesting question, but beyond the scope of this study. The supply with plant-derived carbon from recent photosynthate or storage pools appears to be flexible and the regulation may be important to understand ectomycorrhizal diversity patterns. Since the abundance of most major fungal species did not change after girdling, we assumed that installation of ectomycorrhizal structures may be more energy-consuming than their maintenance. Therefore, it may be more favourable to keep the mycorrhiza than to abandon and re-install them. Investment and running costs should be included in cost-benefit analysis and other modelling exercises to quantify and assess root-mycorrhizal interactions. Economic market models depending on the exchange of 'goods' may be too simplistic (Kummel & Salant 2006).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Supplementary file 1A.** Morphotypes identified by ITS sequencing. Sequences were compared with two databanks, NCBI and UNITE, respectively.

**Supplementary file 1B.** The 16 morphotypes found at roots of beech (*Fagus sylvatica*). Scale bar in cm.

**Supplementary file 1C.** Relative abundance (%) of morphotypes and vital non-mycorrhizal root tips of sunexposed and shade-acclimated beech trees (*Fagus sylvatica*). Root tips were analysed at non-girdled control trees and girdled trees 5 and 10 weeks after girdling. N = non-mycorrhizal root tips. The total number of vital roots was set as 100%. MT numbers refer to morphotypes in Supplementary file 1B.

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THESIS DECLARATIONS

Declarations of the authors own contributions to manuscripts with multiple authors

Chapter 4 Ectomycorrhiza as a link between trees (*Fagus sylvatica*) and belowground food web

- Plant care during the experiment, plant sample preparation and chemical analysis (a part of fine roots), EM harvest, molecular identification and chemical analysis.

Appendix 1 Roots from beech (*Fagus sylvatica* L.) and ash (*Fraxinus excelsior* L.) differentially affect soil microorganisms and carbon dynamics

- Morphological and biomass analysis, sample preparation and $^{13}C$ and $^{15}N$ analysis in plant samples (a part of fine roots), EM harvest, molecular identification and chemical analysis:

Appendix 2 Beech carbon productivity as driver of ectomycorrhizal abundance and diversity

- Analysis of $^{14}C$ allocation on girdled and intact beech saplings. Experimental setup, plant labelling, sample preparation and chemical analysis.

# Plagiarism declaration

To best of my knowledge and belief, this thesis is my own work, all sources have been properly acknowledged, and the assessment task contains no plagiasm.

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