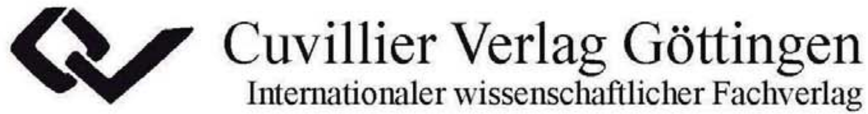
Rodica Pena

Functional diversity of beech (*Fagus sylvatica* L.) ectomycorrhizas with respect to nitrogen nutrition in response to plant carbon supply



Functional diversity of beech (*Fagus sylvatica* L.) ectomycorrhizas with respect to nitrogen nutrition in response to plant carbon supply

Dissertation

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To my beloved grandparents

Rada and Alexandru Ionescu

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Summary

European beech (*Fagus sylvatica* L.) is the dominant tree species of the potential natural vegetation in Central Europe. In temperate forest ecosystems not affected by anthropogenic activities, nitrogen is a growth-limiting factor. Beech trees form mutualistic associations with ectomycorrhizal (EM) fungi, which have the ability to take up different inorganic and organic nitrogen-containing compounds and to improve plant nitrogen-status. EM fungal communities and functions are therefore of major interest for tree nutrition.

In this work, the functional diversity of beech ectomycorrhizas with respect to nitrogen (N) and carbon (C) availability has been investigated. The following hypotheses were tested:

- Mobilization of litter-derived nitrogen by EM fungi differs amongst fungal species in the first phase of litter decomposition.
- Long-distance EM exploration types accumulate more litter-derived N than short distance ones, because of their higher accessibility to the litter.
- Differences in litter-derived N accumulation between EM fungal species decrease over time with the increasing availability of litter-released N via the soil.
- Functional differences exist between EM fungal species with respect to nitrogen uptake and processing.
- EM exhibit positive effects on growth, N uptake and allocation in young beech plants grown under drought conditions. These effects are reduced by long-term C limitation by shading.
- EM fungi influence plant capacity for N uptake by modifications in root architecture.
- EM fungal abundance and diversity in a mature beech stand are independent of current photo-assimilate supply and can be maintained by plant internal resources.

To test these hypotheses, field experiments were set-up in an old-growth beech forest (Tuttlingen, Germany). Furthermore, either experiments were conducted with young beech plants, whose root systems were colonized by typical EM fungal communities, or which were non-mycorrhizal, exposed to light and shade, respectively, and stressed by limiting water availability. The fungal community structure was characterized by diversity indices (Species richness, Eveness and Shannon-Wiener). Morphotyping and ITS sequencing were employed for identification and quantification of EM fungi. ^{15}N was used in the nitrogen uptake studies.

The accessibility of litter-derived N for EM fungal species was investigated over a time scale of 18 months. Mesh bags filled with ^{15}N labelled litter were exposed in the organic soil layer in a beech

forest, and isotopic signatures in soil, mesh bags, fine roots and EM root tips were regularly measured. EM fungal communities were shown to be composed of species with different hyphal length and thus different abilities to directly access litter-derived N. Except *Boletus pruinatus*, a rhizomorphe-forming EM type, the EM fungal species, regardless their exploration type, acquired litter-derived N. In the first phase of litter decomposition, EM fungi competed for litter-derived N in their immediate vicinity and only in a later stage, they probably invaded the litterbag and obtained ^{15}N via external mycelia. In the first phase of litter decomposition, long distance mycelia did not provide an advantage for N acquisition.

To investigate functional diversity of EM fungi for uptake and processing of N, ectomycorrhizal and non-mycorrhizal young beech plants were labelled with ^{15}N-ammonium. ^{15}N and N contents and N turnover in the root tips colonized by different EM fungal species were determined. N turnover in the root tip varied significantly with EM fungal species. Functionality of EM fungal species, with respect to N nutrition, was diminished under drought and shade treatments. The combination of shade and drought, caused a shift of N turnover between non-mycorrhizal and EM root tips. In light, the majority EM root tips were inactive with a turnover close to zero, while in shade combined with drought, they revealed high rates of N turnover, compared with inactive non-mycorrhizal root tips. Furthermore, EM fungal species showed large differences in individual competitiveness under shade and drought stress.

To address questions regarding the effects of EM on N nutrition of young beech plants, growth performance, root architecture and demography, ^{15}N uptake and partitioning between root tips, roots, stems and leaves, total N and C concentrations in above and belowground biomass of EM and NM plants were measured. Colonization of young beech plants by EM fungi had a positive impact on N uptake. Long-term reduction of plant C productivity by shading decreased this effect. EM colonization changed the root architecture under drought conditions, by increasing the number of root tips, root surface area and root length, resulting in improved N uptake.

The relationships between plant C resources and EM fungal diversity in a beech forest were investigated by manipulating carbon flux by girdling. Tree carbohydrate status, root demography and EM fungal colonization were measured repeatedly during one year after girdling. Girdling strongly affects EM fungal community. Despite of maintaining of 90% colonization rate, EM fungal species richness was reduced from about 90 to about 40 taxa. *Cenococcum geophilum*, *Lactarius blennius*, and *Tomentella lapida* were dominant, colonizing about 70% of the root tips, and remained unaffected by girdling. Mainly cryptic EMF species disappeared. EM diversity was positively correlated with glucose, fructose, and starch concentrations of fine roots.

The above results lend supports to the concept of functional diversity of EM fungal species with respect to N nutrition, both by accessing litter-derived N and ammonium. Experiments, in which the carbon flux was modulated by girdling, shading and drought showed that current photo-assimilate supply to EM fungi play an important role in maintaining fungal effectiveness for N uptake. Moreover, in a typical beech forest, EM fungal diversity was strongly affected by reduction in belowground carbon allocation. Beech maintains numerous rare EM fungal species by recent photosynthate. These EM fungi may constitute biological insurance for adaptation to changing environmental conditions.

Zusammenfassung

Die Rotbuche (*Fagus sylvatica* L.) ist die dominierende Baumart der potentiell natürlichen Vegetation Mitteleuropas. In temperaten Waldökosystemen, welche nicht durch anthropogene Aktivitäten beeinflusst werden, ist Stickstoff ein wachstumslimitierender Faktor. Zur Verbesserung des Stickstoffhaushalts bilden Buchen mutualistische Interaktionen mit Ektomykorrhizapilzen (EM) aus die Fähigkeit zur Aufnahme von unterschiedlichen anorganischen und organischen Stickstoffverbindungen haben. Daher sind EM Gesellschaften und deren Funktionen von großem Interesse für den Nährstoffhaushalt der Bäume.

Diese Arbeit befasst sich mit der funktionellen Diversität von Buchen-Ektomykorrhiza, im Hinblick auf die Stickstoff- und Kohlenstoffverfügbarkeit. Folgende Hypothesen wurden getestet:

- Die Mobilisierung von Stickstoff aus Laubstreu unterscheidet sich zwischen EM Pilzarten während der ersten Phase der Zersetzung.

- Die EM-Pilze des Explorationstyps „lange Distanz“ akkumulieren, aufgrund ihrer besseren Erreichbarkeit mehr streubürtigen Stickstoff als der Explorationstyp „kurze Distanz“.
- Die Unterschiede in der Aufnahme von Stickstoff aus Laubstreu zwischen EM-Pilzarten verringern sich im Laufe der Zeit mit der steigenden Verfügbarkeit von freigesetztem Stickstoff im Boden.
- Es existieren funktionelle Unterschiede zwischen EM-Arten hinsichtlich ihrer Stickstoffaufnahme und –umsetzung.
- Die Effektivität der Stickstoffernährung von EM-Gesellschaften hängt von ihrem laufenden Bedarf an Photoassimilaten ab.
- EM-Pilze haben einen positiven Effekt auf das Wachstum, die Stickstoffaufnahme und –verteilung in jungen Buchen, die unter Trockenstressbedingungen wachsen. Diese Effekte sind lichtabhängig.
- EM-Pilze beeinflussen die Kapazität der Pflanze zur Stickstoffaufnahme durch Modifizierungen in der Wurzelarchitektur.
- Abundanz und Diversität von EM-Pilzarten in Buchenaltbeständen sind unabhängig von der aktuellen Verfügbarkeit von Photoassimilaten und können durch interne pflanzliche Ressourcen aufrechterhalten werden.

Um diese Hypothesen zu testen, wurden Feldexperimente in Buchenaltbeständen (Tuttlingen, Deutschland) durchgeführt. Zusätzlich wurden Experimente mit jungen Buchen, deren Wurzelsystem entweder mit typischen EM-Gesellschaften kolonisiert (EM) oder nicht mykorrhiziert (NM) waren,

durchgeführt. Diese wurden Licht bzw. Schattierung und zusätzlich Trockenstress ausgesetzt. Die Struktur der pilzlichen Gesellschaft wurde durch Diversitäts-Indizes (Artenzahl, Eveness und Shannon-Wiener-Index) charakterisiert. Zur Identifikation und Quantifizierung der EM-Pilze wurden morphotypische Merkmale und ITS-Sequenzierung genutzt. Für die Untersuchung der Stickstoffaufnahme wurde ^{15}N eingesetzt.

Zudem wurde der Aufschluss von Stickstoff aus Buchenlaub durch EM-Pilzarten über einen Zeitraum von 18 Monaten untersucht. Hierzu wurden Beutel mit einer definierten Maschenweite, die mit ^{15}N markierter Streu gefüllt waren, in die Humusauflage in einem Buchenwald eingebracht. Regelmäßig wurde die isotopische Signatur im Boden, im Laubbeutel, sowie in Feinwurzeln und EM-Wurzelspitzen gemessen. Es wurde gezeigt, dass EM-Gesellschaften Pilzen Arten mit unterschiedlichen Fähigkeiten zum Aufschluss von streubürtigem Stickstoff enthalten. Mit Ausnahme von *Boletus pruinatus*, einer Rhizomorph-bildendenden EM-Art, nahmen die EM-Pilze Stickstoff aus Buchenstreu unabhängig von ihrem Explorationstypen auf. In der ersten Phase der Streuzersetzung konkurrierten EM-Pilze in ihrer unmittelbaren Umgebung um den aus der Streu freigesetzten Stickstoff. Erst in einer späteren Phase der Zersetzung drangen die Pilze mit externen Hyphen in die mit markiertem Streu gefüllten Beutel ein und nahmen ^{15}N auf. Daraus kann geschlossen werden, dass in der ersten Phase der Zersetzung des Streuabfalls ein weitreichendes Myzel keinen Vorteil bei der Akquisition von Stickstoff bietet.

Um die funktionelle Diversität von EM-Pilzen hinsichtlich der Aufnahme und Umsetzung von Stickstoff zu untersuchen, wurden mykorrhizierte und nicht mykorrhizierte junge Buchen mit ^{15}N-Ammonium markiert. In mit unterschiedlichen EM-Pilzarten mykorrhizierten Wurzelspitzen wurde ^{15}N- und Gesamt-N-Gehalt, sowie der Stickstoffumsatz, bestimmt. Letzterer zeigte signifikante Unterschiede zwischen mit verschiedenen EM-Arten kolonisierten Wurzelspitzen. Die Funktionalität von EM-Pilzarten hinsichtlich der Stickstoffernährung verringerte sich bei Trockenstress und Schattierung. Die Kombination von Schattierung und Trockenheit führte zu einer Veränderung des Stickstoffumsatzes zwischen NM und EM Wurzelspitzen. Bei Schattierung war ein Großteil der mykorrhizierten Wurzelspitzen inaktiv. Bei der Kombination von Schattierung und Trockenheit zeigten sich an mykorrhizierten Wurzelspitzen höhere Stickstoffumsatzraten im Vergleich zu inaktiven, nicht mykorrhizierten Wurzelspitzen. Des Weiteren wiesen EM-Arten große Unterschiede in ihrer individuellen Konkurrenzfähigkeit bei Schattierung und Trockenstress auf.

Um Fragen hinsichtlich der Effekte von EM auf die Stickstoffversorgung junger Buchen, deren Wachstum sowie ihrer Wurzelarchitektur und Demographie zu beantworten, wurden Aufnahme und Aufteilung von ^{15}N zwischen Wurzelspitzen, Wurzeln, Stamm und Blättern, sowie die Stickstoff-

und Kohlenstoffkonzentration in ober- und unterirdischer Biomasse von EM und NM Pflanzen gemessen. Die Kolonisierung von jungen Buchen durch EM-Pilze hatte einen positiven Einfluss auf die Stickstoffaufnahme. Die langfristige Reduzierung von pflanzlicher Kohlenstoffproduktivität, bedingt durch Beschattung, verringerte diesen Effekt. Unter Trockenstressbedingungen veränderte die EM-Kolonisierung die Wurzelarchitektur durch eine erhöhte Anzahl an Wurzelspitzen, die Vergrößerung der Wurzeloberfläche und -länge und führte damit zur Verbesserung der Stickstoffaufnahme.

Der Zusammenhang zwischen pflanzlichen Kohlenstoffres Sourcen und EM-Diversität in Buchenwäldern wurde durch eine Manipulation des Kohlenstofftransportes mittels Ringelung untersucht. Der Kohlenhydratstatus des Baums, die Wurzeldemographie und die EM-Kolonisierung wurden wiederholt in einem Zeitraum von einem Jahr nach der Ringelung analysiert. Die Ringelung hatte einen großen Einfluss auf die EM-Gesellschaft. Ungeachtet der Aufrechterhaltung einer Kolonisierungsrate von 90 %, wurde der EM-Artenreichtum von 90 auf 40 Taxa verringert. *Cenococcum geophilum, Lactarius blennius* und *Tomentella lapida* waren die dominierenden Arten, die über 70 % der Wurzelspitzen kolonisierten und durch die Ringelung nicht beeinflusst wurden. Hauptsächlich kryptische EM-Arten verschwanden. Die EM-Diversität korrelierte positiv mit der Glukose-, Fruktose- und Stärkekonzentration von Feinwurzeln.

Die oben genannten Ergebnisse unterstützen das Konzept der funktionellen Diversität von EM-Arten hinsichtlich der Stickstoffversorgung, sowohl durch die Bereitstellung von Stickstoff als auch von Ammonium aus Streu. Experimente, in denen der Kohlenstoffumsatz durch Ringelung, Schattierung und Trockenheit verändert wurde zeigten, dass die aktuelle Versorgung der EM-Pilze mit Photoassimilaten eine wichtige Rolle in der Effektivität der Pilze zur Aufrechterhaltung einer Stickstoffversorgung spielt. In einem typischen Buchenbestand war darüber hinaus die EM-Diversität stark durch Reduktion der unterirdischen Kohlenstoffallokation beeinflusst. Die Buche hält auch die Symbiose mit verschiedenen, seltenen EM-Arten durch aktuelle Photosyntheseprodukte aufrecht. Diese EM-Arten bilden vermutlich eine biologische Absicherung zur Anpassung an sich verändernde Umweltbedingungen.

CHAPTER 1: Mycorrhizal community structure and nutrient supply

1.1. Mycorrhizal mutualism

Mycorrhiza is the mutualistic association between certain soil fungi and plant roots: while the fungus improves plant mineral nutrition, the plant supplies the fungus with carbohydrates, which are ultimately derived from photosynthesis (Smith and Read 2008). More than 90% of the world's plants have mycorrhizal roots (Trappe 1987). There are seven types of mycorrhizal associations: arbuscular Mycorrhiza (AM), ectomycorrhiza (EM), ectendomycorrhiza, arbutoid mycorrhiza, monotropoid mycorrhiza, ericoid mycorrhiza, and orchid mycorrhiza (Smith and Read 2008). EM associations are the predominant form in temperate forests due to the dominance of members of *Pinaceae*, *Fagaceae*, *Betulaceae* and *Salicaceae,* forming preferentially EM (Brundrett 2004). Other kinds of mycorrhizas such as ericoid, ectendomycorrhiza and AM exist also in temperate forest ecosystems, but they will not be considered in this thesis, since the research here is focused on European beech (*Fagus sylvatica* L.).

1.1.1. Ectomycorrhizas: functional characteristics

There are three characteristic structures of EM symbiosis: the mantle, consisting of hyphae ensheating the root tips (Figure 1.1C), the hyphal net (Harting net) formed in the intercellular space of root epidermis and cortex cells, which maximizes the contact between plant and fungus (Figure 1.1D), and the external mycelium formed by single or aggregated hyphae that extend into the surrounding soil from the surface of the mantle (Figure 1.1C). In contrast with EM root tip, non-ectomycorrhizal (NM) root tips show root hairs (Figure 1.1A, B).

The emanating hyphae of EM increase the potentially absorbing surface area of the roots (Smith and Read 2008). In some cases, these hyphae may form vessel-like structures called rhizomorphs that are capable of nutrients and water transport along long distances (Duddridge et al. 1980, Agerer 2001). External mycelia differ greatly among EM fungal species (Agerer 2001). Based on characteristics such as structure, abundance and lengths of external mycelia, Agerer (2001) has classified EM fungi after four main exploration types: contact, short-distance, medium-distance, and long-distance type, respectively. Contact exploration type EMs possesses a smooth mantel with no or very few emanating hyphae; the rhizomorphs are absent. Short-distance exploration types EMs have usually short, but dense emanating hyphae; the rhizomorphs are lacking. Fungal species characterized as medium-distance exploration type may form rhizomorphs and the hyphae are more extended in the surrounding soil than those of the short-distance exploration type. Long-distance type EMs are

characterized by a smooth mantle with highly differentiated rhizomorphs. In beech forests, the most frequent EM species belong to all different classes of exploration types (Table 1.1).

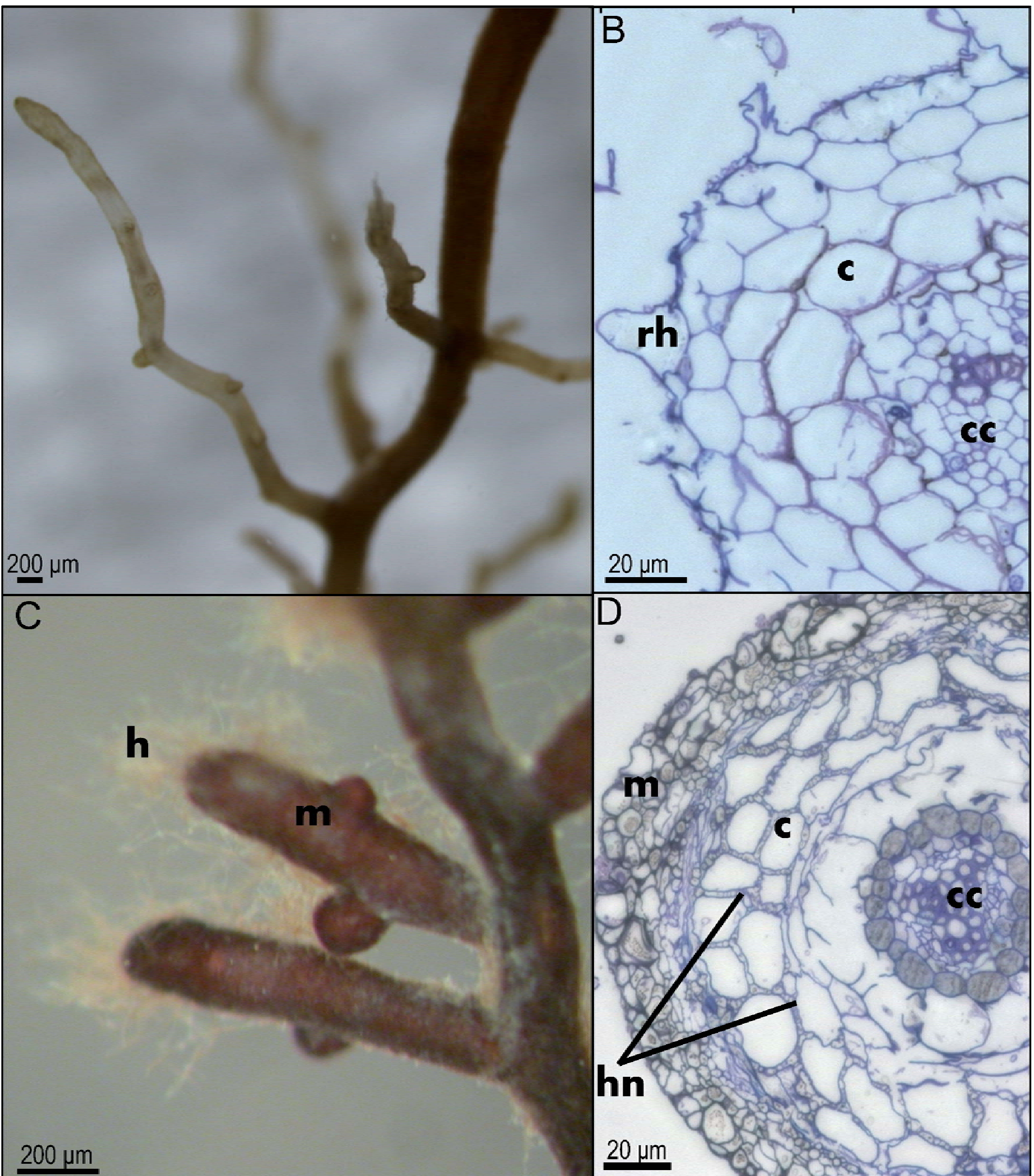

Figure 1.1: Non-mycorrhizal (A, B) and Ectomycorrhizal (C, D) root tips of young beech. Cortex cell (c), central cylinder (cc), hyphae (h), Hartig net (hn) rh, root hair (rh), mantle (m).

Table 1.1: Classification of the most frequent EM fungal species associated with beech roots (Buee et al. 2005, Pena et al. 2010) according to their mycelia system and their putative exploration type (Agerer 2001, Courty et al. 2008)

Species	Exploration type
Lactarius blennius	Contact
Lactarius subdulcis	Contact
Russula sp	Contact
Tomentella lilacinogrisea	Contact
Tomentella subclavigera	Contact
Cenococcum geophilum	Short-distance
Hebeloma crustuliniforme	Short-distance
Clavulina cristata	Medium-distance
Cortinarius sp	Medium-distance
Boletus pruinatus	Long-distance

1.1.2. Ectomycorrhizal communities

A unanimously accepted characteristic of EM fungal communities is their high diversity (Horton and Bruns 2001, Dahlberg 2001, Rinaldi et al. 2008). The EM status has been proven for thousands of fungal species, but exact estimates about EM fungal species richness are still not possible (Rinaldi et al. 2008). In a recent review, Rinaldi et al. (2008) estimated the contemporary known species richness of EM fungi of 7750 species and predicted these numbers to increase to 20000 to 25000 species, with the advent of sequencing of environmental samples.

In typical boreal and temperate forest ecosystems, fine roots of trees are almost 100% colonized by EM fungi (Smith and Read 2008). About 60 to 90 EM fungal species were found in a single stand of mature beech trees (Buee et al. 2005, Pena et al. 2010). EM fungi colonize rapidly, within days after their emergence, all orders of lateral roots formed at the axis of long roots with unlimited growth (Smith and Read 2008). If the laterals emerge from an already colonized parent root, they will be usually colonized with the same fungus. In contrast, if laterals originate from a non-colonized long root or if they belong to a recently germinated seedling with a completely new root system, competition between the available fungi in the soil occurs. In the latter case, EM formation depends on events such as recognition and compatibility, combined with direct inter- and intra-specific competition (Koide et al. 2005).

The structure of the EM community is influenced by abiotic and biotic factors (Bruns 1995, Koide et al. 2005). Soil properties like stratification, moisture, temperature, or fertility, but also the interactions among species can contribute to EM fungal diversity and may limit a species to a certain niche. Differences in substrate preference might be related to different functionality or life strategies of EM fungal species (Bruns 1995).
To date, knowledge on functional diversity of EM fungi in forests are scarce (Jones et al. 2009). In this thesis, functions of EM communities and individual EM species in field communities and experimental systems will be addressed.

1.1.3. Measuring EM community structures

Until the last decade, EM communities were almost exclusively described by the occurrence and abundance of sporocarps (Gardes and Bruns 1996). It was assumed that the production of sexual structures mirrors the relative abundance of vegetative structures of different species (Smith and Read 2008). But it became apparent that this approach did not represent complete EM communities, because very important EM fungi, e.g., *Cenococcum geophilum* and species of the *Corticinaceae* and *Thelephoraceae* have no or inconspicuous sporocarps (Gardes and Bruns 1996, Taylor and Bruns 1999, Peter et al. 2001a, Peter et al. 2001b). Therefore, the study of EM fungal communities at the level of root tips was necessary. To characterize EM communities, morphological description of mantle and extraradical mycelium, so-called morphotyping, has been and is being applied (Agerer 1987-1989, Agerer 1991). However, this technique has critical limitations because it is time consuming and requires an experienced investigator. Furthermore, because of high morphological similarity among different EM species, morphotyping is sometimes inaccurate and one EM species can have different morphotypes depending on age or host plant (Agerer 1987-1989, Peter et al. 2001b).
The precision of fungal identification has been greatly improved through the development of molecular techniques and the use of DNA sequence databases (Taylor and Bruns 1999, Horton and Bruns 2001). Sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA are currently most used as barcoding target for fungal identification (Ryberg et al. 2009). The ITS region is situated between the small subunit (SSU) and the large subunit (LSU) ribosomal RNA (rRNA), contains two non-coding spacer regions separated by 5.8S rRNA, and has a size of 650-900 bp (Gardes and Bruns 2001). These non-coding spacer regions are characterized by a fast rate of evolution, resulting in high sequence variation between closely related species (Anderson and Cairney 2004). White et al. (1990) designed the first PCR primers for amplification of ITS regions

from fungal DNA. The primer pair ITS1 and ITS4 (White et al. 1990) are still widely used today, but they display a lack of specificity for fungal template DNA and co-amplifiy angiosperm DNA. Thus from a mixed plant-fungal DNA sample, plant DNA can be also amplified. To improve specificity, several taxon-specific primers such as ITS1f or ITS4b that allow selective amplification of basidiomycete DNA from mixed DNA samples extracted from mycorrhizal root tips have been described (Gardes and Bruns 1993).

To date, identification of EM fungi is achieved by comparison of ITS sequences with known sequences deposited in public databases (Koljalg et al 2005, Ryberg et al. 2009). As DNA extraction and amplification may strongly vary amongst fungal species (Tedersoo et al. 2010), currently the most reliable procedure to study EM community composition is a combination of morphotyping and Sanger sequencing. More recent studies describe and compare complex soil fungal communities using high-throughput sequencing techniques (Buee et al. 2009). However, these methods still suffer from a relatively poor accuracy and require careful selection of molecular tools (Tedersoo et al. 2010).

In the present thesis, a combination of morphotyping and ITS sequencing was employed to investigate EM communities. This has the advantage that in addition to an accurate identification, the frequency of the fungi can also be reliably determined. These measures are required to determine not only the species richness but also diversity indices such as the Shannon-Wiener or Evenness (Dahlberg 2001).

1.2. Functions of EM in C and N exchange between plant and soil

1.2.1. Carbon transfer to EM fungi

A vast range of EM fungi show saprotrophic abilities (Koide et al. 2008, Courty et al. 2005) and can exist in the absence of the host plants freely in the soil. Formation and sustainability of EM symbiosis strongly depend on plant derived carbon (C) supply to the associated fungi (Smith and Read 2008). The transfer of current assimilates to soil microorganisms has been estimated using different approaches such as short pulse ^{13}C labelling (Högberg et al. 2008, Rühr et al., 2009), concomitant measurements of ^{13}C isotopic signature of CO_2 respired and newly assimilated organic matters pools (Gessler et al. 2007, Kodama et al. 2008), as well as by different experiments, e.g. by disruption of the photosynthate flux by tree girdling (Högberg et al. 2001, Högberg et al. 2002, Bhupinderpal-Singh et al. 2003. Pena et al. 2010) or by application of free air carbon dioxide enrichment (FACE), with a different isotopic signature to identify new tree derived soil C (Godbold

et al. 2006). All these studies have shown that the recent assimilates are rapidly transferred to the belowground compartment and much of this C flux becomes available to EM fungi. A field-scale girdling experiment in a conifer forest (Högberg et al. 2001) showed that by interruption of C flux, soil microbial respiration decreased in two weeks by 56%, of which 41% was due to the loss of EM mycelia (Högberg et al. 2001, Högberg et al. 2002). In another study, the contribution of EM external mycelium and of roots to forest soil CO_2 efflux have been estimated to account ca. 25 % and 15 %, respectively and the residual 60 % were due to soil heterotrophic respiration (Heinemeyer et al. 2007). Furthermore, CO_2 enrichment in a poplar plantation indicated that EM fungal C constitutes the dominant (62%) C input to soil organic matter (Godbold et al. 2006). Controlled experiments using ^{13}C isotope signatures indicated that as much as 22% of photosynthate was allocated to EM fungi (Hobbie 2006).

Monitoring of EM fungal communities, colonization rate, and formation of fruiting bodies showed negative effects of reduced plant C supply emphasizing the strong C demand of EM fungi. For example, when C flux to roots was interrupted by girdling, or reduced by defoliation, EM fruiting body formation ceased (Högberg et al. 2001, Kuikka et al. 2003). Defoliation of conifers also suppressed EM morphotypes with thick mantels compared to those with thin mantels (Markkola et al. 2004), and increased the number of root tips colonized by low-biomass EM that probably require less plant derived C (Saravesi et al. 2008). Reduction of photosynthesis by long-term shading resulted in a strong diminution of EM root colonization compared with sun-exposed beech trees (Druebert et al. 2009). In conclusion, these studies have shown that EM have important ecological roles in belowground C allocation and that plant C productivity is an essential driver of EM formation. However, large gaps exist regarding the importance of plant C flux and C-storage pools for EM diversity and functions. Since drought is an important environmental factor that has strong effects on photosynthesis and belowground C allocation, it is of particular interest if and how EM fungal communities and functions respond to this stress factor. This question is particularly relevant for beech, a species known to be relatively drought sensitive (Ellenberg and Strutt, 2009). Therefore, in this thesis the C-flux was modulated by girdling, shading and drought to study EM functions and community composition.

1.2.2. Nitrogen forms and plant availability

Nitrogen (N) is an essential constituent of many organic compounds of plant cells, such as amino acids, amides, proteins, nucleic acids, pigments, coenzymes etc. For growth and optimal physiological functioning, plants require a permanent supply with N. Although the atmosphere

contains about 386 x 10^{13} t (78% by volume) dinitrogen (N_2), N is a limiting factor for plants in most near-natural forest ecosystems (Rennenberg 1998, Chen et al. 2000), since N_2 cannot be directly assimilated by plants (Semoka 2008). In soil, N occurs in complex organic compounds such as humic acids, which also are not available for most plants. Only a small fraction of total N in the soil is present in forms such as ammonium (NH_4^+), nitrate (NO_3^-), or amino acids that can readily be taken up by plant roots. Apart from anthropogenic N pollution, the most important source of N in forest ecosystems is leaf litter (Chen et al. 2000). In the first phase after shedding, the litter is decomposed by soil microorganisms, mainly saprotrophic fungi (Figure 2). Thereby, N is transferred into the dissolved organic N pool and then mineralized to NH_4^+ by other microorganisms, mainly bacteria (Scharenbroch and Lloyd 2004). NH_4^+ can either be taken up by plant roots, by soil microorganisms, including EM fungi, or by chemoautotrophic nitrifiers (Figure 1.2). Nitrifying bacteria, *Nitrosomonas* spp and *Nitrospira* spp, oxidize NH_4^+ to nitrite (NO_2^-) and *Nitrobacter* ssp further oxidize NO_2^- to NO_3^- through the process of nitrification (Figure 1.2, Scharenbroch and Lloyd 2004).

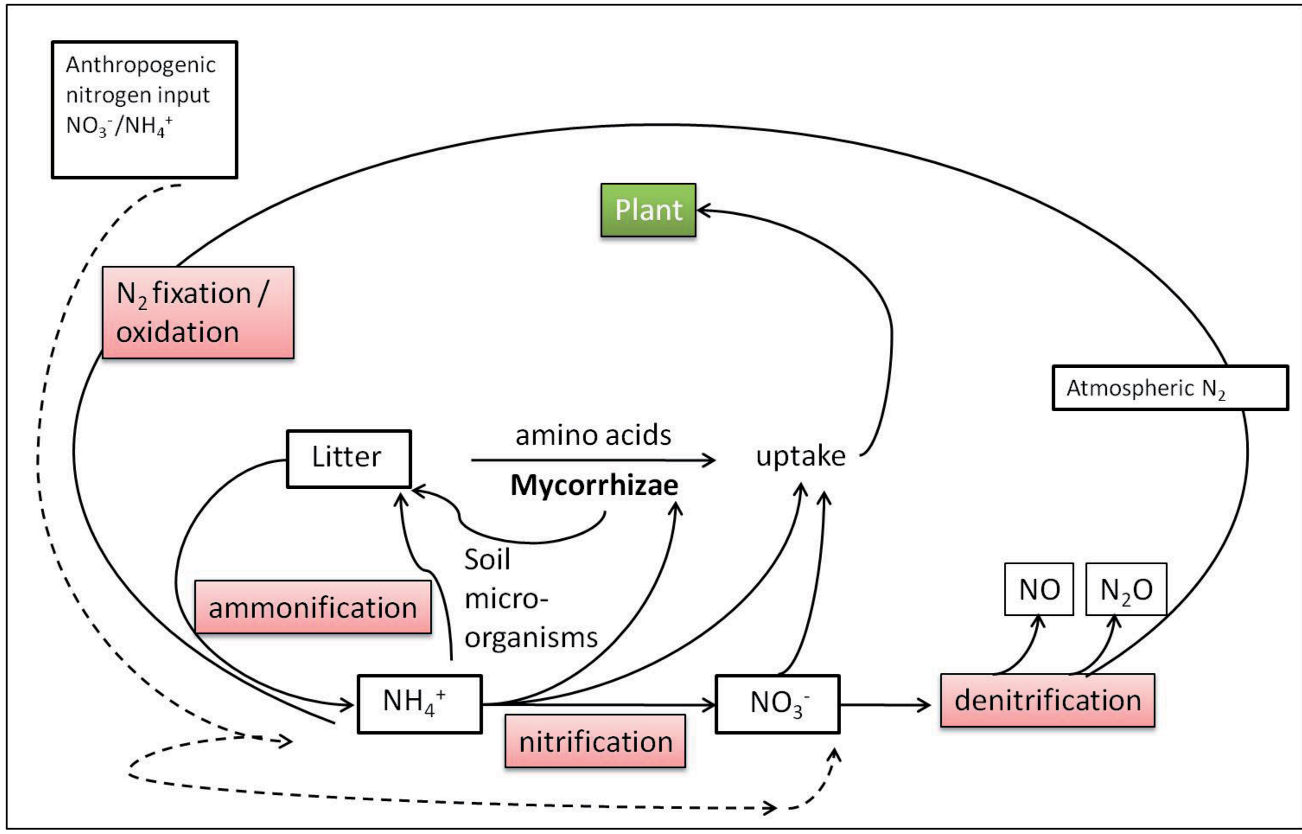

Figure 1.2: Major pathways of nitrogen cycle in forest ecosystems were displayed. Adapted after Schulze (2000).

N mineralization rate depend on various abiotic and biotic factors such as temperature, humidity, physical and chemical characteristics of the soil, microbial populations, and quantity and quality of

litter (Yang et al. 2003). For instance, the rate of mineralization increases at higher temperatures and intermediate soil water content (Brüggemann et al. 2005). Low soil water content inhibits microbial activities by reducing diffusion of soluble substrates (Schjonning et al. 2003), microbial mobility (Killham et al. 1993), and intracellular microbial water potential (Stark and Firestone, 1995). C and N contents of the organic material are also factors that influence N mineralization. At a high C:N ratio, the microorganisms are N-limited, while below a critical value they are C-limited (Kaye et al. 1997). In the latter case, N immobilization in heterotrophic microorganisms is restricted, resulting in formation of inorganic N (Kaye et al. 1997). In the present study, we have modulated belowground C availability by girdling, drought and sugar feeding (Winkler et al. 2010, see appendix 2). Girdling resulted in a significant increase of N mineralization, an effect that was combated by glucose application. C limitation facilitated in our study high soil NO_3^- concentrations, and by application of glucose, microbial NH_4^+ immobilization was promoted. Soil NH_4^+ concentrations increased in response to drought, also.

Organically bound N may represent an N source for many plant species in arctic, boreal, temperate, Mediterranean shrubland and alpine ecosystems (Näsholm et al. 2009). Organic N sources that are directly accessible for plants are free amino acids in soil solution, whereas the organic N pool is mainly formed by peptide- and protein-bound amino acids (Näsholm et al. 2009). Therefore, an intermediate step in plant organic N nutrition is depolymerization of organic macromolecules to plant available monomers (Schimel and Bennett 2004). The main pathway for this is the proteolysis of peptides and proteins. The involved proteolytic enzymes are mainly produced by soil microorganisms including mycorrhizal fungi (Näsholm et al. 2009). Although it is known that EM fungi produce different exoenzymes for degradation of organic nutrients (Read and Perez-Moreno 2003), we have little information if this results in functional diversity with respect to mycorrhizal N –accumulation. To address this question in the present study, beech leaf litter, which is degraded very slowly, was used to follow N uptake by different EM species in an old-growth forest.

1.2.3. Nitrogen in forest soils and uptake by ectomycorrhizal fungi

In forest soils, NH_4^+ is the dominant form of inorganic N (Nadelhoffer et al. 1984, Semoka 2008), because nitrification is usually suppressed by low soil temperature, low pH, and allelopathic soil conditions (Kronzucker et al. 1995). However, soil NH_4^+ to NO_3^- ratios vary strongly with forest type, time point, and environmental conditions (Nadelhoffer et al. 1985). In undisturbed forest soils the ratio is about 10:1, but it might be higher in deciduous (33:1) than in coniferous forest (about 5:1, Jerabkova et al. 2006). Over a range of deciduous forest soils, NH_4^+ concentrations varied between

2.4 and 12.5 mg kg^{-1} and NO_3^- from <0.1 to 2.8 mg kg^{-1} (Vitousek et al. 1982). Soil NO_3^- concentrations increased after disturbances such as fire, wind throw, and clear cutting (Kronzucker et al. 1995).

The study site in this thesis was a beech forest located in a low mountain range in southern Germany, Tuttlingen forest. In the investigated soil, NH_4^+ was the dominant form of inorganic N. NH_4^+ concentrations ranged from 4 to 8 mg kg^{-1}, while NO_3^- were less than 2 mg kg^{-1} (Dannenmann et al. 2009, see appendix 1). DON concentrations ranged between 20 and 40 mg kg^{-1}(Dannenmann et al. 2009, see appendix 1). All these N sources of variable sizes may be accessed by beech trees via their fungal symbionts, since the roots of European beech, like those of all *Fagaceae*, are almost 100% colonized by EM fungi (Smith and Read 2008).

EM fungi may endow plants access to organic forms of N, inducing a shortcut in the N cycling. The abilities of EM fungi alone or, in symbiosis, to use different organic N sources such as amino acids or proteins were extensively investigated in laboratory studies (Harley and Smith 1983, Abuzinadah and Read 1986, Baar et al. 1997, Chalot et al 1998, Gobert and Plassard 2008). In the field, proteolytic capacities of EM fungi were indirectly assessed by measuring the natural abundance of ^{15}N in fungal fruiting bodies (Gebauer and Dietrich 1993, Taylor et al. 1997). Greater ^{15}N contents in EM fruiting bodies indicate higher abilities to access organic than mineral N sources (Lilleskov et al. 2002). By employing ^{15}N and ^{13}C labeled amino acids, the ability of EM fungi to take up amino acids was shown (Schimel and Chapin 1996, Näsholm et al. 1998, McKane et al. 2002). We have shown recently a positive correlation of *Cenococcum geophilum* abundance with glutamine uptake by mature beech (Dannenmann et al. 2009, see appendix 1). Under field and also under the green house conditions beech trees preferred amino acids over inorganic N (Dannenmann et al. 2009, Winkler et al. 2010, see appendix 1,2). Same EM fungi prefer NH_4^+ to NO_3^- (Finlay et al. 1989, Ek et al. 1994, Rangel-Castro et al. 2002, Guidot et al. 2005). For example, uptake and assimilation rates were two fold higher for ^{15}N-labelled NH_4^+ than for NO_3^- in *Fagus sylvatica* colonized by *Paxillus involutus* (Finlay et al. 1989). But, there are also EM fungi, such *Hebeloma cylindrosporum* that grow better with NO_3^- as source of N than with NH_4^+ (Scheromm et al. 1990).

Despite of the well known involvement of EM fungi in N uptake, there are scarce data that build a link between plant C status, EM fungi and N nutrition. Usually, the questions about functionality of EM fungi were addressed in laboratory studies, where the aspect of functional diversity has been overlooked.

1.3. Occurrence and N requirements of European beech

Fagus sylvatica L. is the most important deciduous species of the potential natural vegetation in forests of Central Europe (Ellenberg and Strutt, 2009). Beech dominates the vegetation in the submontane and montane belt from 200 m above the sea level (asl) in the northern and from 500 asl in the southern part of Europe up to an altitude of 1200 to 1400 m asl. At the montane level, beech trees mix with conifers (Ellenberg and Strutt, 2009). European beech requires a suboceanic to weakly subcontinental climate. It grows on all types of bedrocks, but a preference for calcareous soil of pH 5.7 – 7.4 was observed (Ellenberg and Strutt, 2009).

In the juvenile phase, beech trees are very shade–tolerant (Gansert and Sprick 1998), but when increased irradiance is available, e.g. in forest gaps, the growth of young beech plants is stimulated (Tognetti et al. 1994, Johnson et al. 1997, Tognetti et al. 1998, Collet et al. 2001, Aranda et al. 2004, Parelle et al. 2006). Ellenberg and Strutt (2009) identified water availability as the most decisive factor for the habitat requirements of beech. Compared with other temperate broad-leaved species, European beech is more sensitive to drought. Growth and the competitive ability of beech decrease strongly during intensive drought periods that may occur during the growth phase (Peuke et al. 2002, Gessler et al. 2007). When water availability is moderate, beech prevents strong reduction in turgor, leaf water potential and in photosynthesis by stomatal regulation (Backes and Leuschner 2000). However, during severe drought stomatal closure is not sufficient to avoid decreases in predawn leaf water potential, photosynthetic capacity, hydraulic conductivity (Gessler et al. 2001), canopy conductance (Granier et al. 2000, Gessler et al. 2004) and growth (Braun and Flückiger 1987, Lebourgeois et al. 2005). Drought influences directly the water status of the beech and impairs pedospheric N uptake, thus affecting N metabolism (Gessler et al. 2004).

The nutritional status of trees is generally characterized by their foliar nutrient concentrations. In field grown beeches in North and Central Europe, leaf N concentrations range from 24 to 28 mg g^{-1} dry mass (Balsberg-Pahlsson 1988, Duquesnay et al. 2000). High N concentrations in leaves appeared in the spring, summer months during leaf growth, and decreased due to N retranslocation in autumn (Santa Regina and Tarazona 2001). Drought treatment had no significant effect on N concentrations in leaves and roots of young plants (Fotelli et al. 2002b, Peuke and Rennenberg 2004), but reduced N concentration in stems (Peuke and Rennenberg 2004).

As outlined above, under field conditions beech trees prefer NH_4^+ uptake to NO_3^-. Even if beech may have the capacity to take up NO_3^-, the ratios NH_4^+ to NO_3^- found in soil of deciduous forests of about 10:1 inhibit NO_3^- uptake (Gessler et al. 1998a). Furthermore, beech trees display a seasonal and a

diurnal course of NH_4^+ net uptake (Gessler et al. 1998a, Gessler et al. 2002). The rates of NH_4^+ uptake have a maximum in midsummer and lower values in spring and autumn and increase with soil temperature (Gessler et al. 1998a). The diurnal patterns of NH_4^+ uptake are 50% higher during the day than during the night and the highest rates are observed during midday and in the afternoon (Gessler et al. 2002). Drought decreases N uptake rates of beech seedlings and the effects are aggravated by the presence of competitors such as *Rubus fruticosus* (Fotelli et al. 2002b).

Gessler et al. (2007) evaluated the potential risks for European beech under climate-change conditions, characterized by weather extremes with frequent drought periods (IPCC 2001). They concluded that growth and competitive ability of beech might be reduced under predicted climate conditions. Mainly seedlings, but also the adult trees may suffer from restricted nutrient uptake capacity under limited water availability (Gessler et al. 2007). Given these interrelationships between drought, C production and N nutrition, the role played by EM fungi has to be better understood.

1.4. Objectives of the present thesis

This thesis focuses on functional diversity of beech ectomycorrhizas. The overarching goal was 1) to investigate the functions of different EM fungal species with respect to N uptake and 2) to estimate the significance of plant C supply to sustain EM diversity.

To address these aims the following hypotheses were tested:

- In a mature beech forest, the EM community is composed of species with different abilities to access liter-derived N. In particular, we assumed that EM of the long distance exploration type accumulate litter-derived N faster than short distance type ones, because they can explore a wider range and reach N resources inaccessible to roots. Differences in ^{15}N accumulation between EM fungal species decrease over time with the increasing availability of litter-released N via the soil.

  To test these hypotheses ^{15}N labeled beech leaf litter was exposed in the top soil of an old growth beech forest. ^{15}N enrichment was regularly measured in soil, fine roots and root tips colonized by different EM fungal species. To distinguish between N uptake from litter patches and from the soil, a set of litterbags were removed after 14 months and the incorporation of the label in EM root tips and roots in the presence and absence of the litterbags was measured **(Chapter 2).**

- EM fungi reveal functional diversity with respect to N transport and turnover. Under environmental conditions such as low irradiance and restricted water availability they reveal complementary behavior rather than competition.

  To test this hypothesis, we conducted a growth chamber experiment with young beech plants, whose root systems were either colonized by typical EM fungal communities, or which were non-mycorrhizal (NM). We expected that variation in the environmental conditions would result in similar, possibly negative effects, on N transport and turnover if the whole EM community showed functional redundancy or, in contrast functional complementary would result in differential behavior of different EM species in response to changing light and water supply **(Chapter 3).**

- N uptake by EM plants is higher than in NM plants under drought stress and lower under shade because of differences in C allocation and C costs.

  To test this hypothesis, the performance of young beech plants, whose root systems were non-colonized or were colonized by typical EM fungal communities, was tested under different light and water supply. It was assumed that N-uptake was maintained in EM under drought stress but that shade would impose C-limitation, thereby preventing maintenance of EM-based N influx **(Chapter 4).**

- EM fungal abundance and diversity are independent of the current photosynthates supply and can be maintained by internal resources.

  To investigate this assumption, old-growth beech trees (*Fagus sylvatica* L.) were girdled to suppress carbon allocation to roots. Tree carbohydrates status was measured repeatedly during one year and related to root demography, EM colonization and EM species abundance **(Chapter 5).**

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CHAPTER 2: How accessible is beech litter-derived nitrogen for different ectomycorrhizal fungal species

2.1. Introduction

In temperate forest ecosystems, nitrogen (N) is a major limiting nutrient (Vitousek et al. 2002). Litter accumulated on the forest floor represents potentially the main source of N required for plant growth (Hart and Firestone 1991). N availability for plants was traditionally considered dependent on conversion of organically bound N forms to inorganic ones by mineralization mediated by decomposer organisms in the soil (Boberg et al. 2010). To date, it is known that plants can also obtain N from organic sources by ectomycorrhizal (EM) fungi (Perez-Moreno and Read 2000, Brearley et al. 2003, Chapman et al. 2006, Wallander et al. 2006).

In temperate and boreal forests, the vast majority of the root tips are colonized by EM fungi (Smith and Read 2008). Forest trees are primarily supplied with N via EM fungi (Lindahl et al. 2002). EM fungi have capacity to use organic N from complex organic substrates (Näsholm et al. 1998) because they contain the necessary hydrolytic enzymes (Martin et al. 2008, Martin et al. 2010). However, different EM fungal species have different enzymatic activities (Abuzinadah and Read 1986, Courty et al 2005, Buee et al. 2007). Furthermore, the enzymatic activity patterns vary among species which coexist in the same ecological niche and also change within species between niches (Buee et al. 2007). For instance, in soil organic layer *Lactarius quietus* and *Tomentella* sp. expressed strong cellobiohydrolase activities, while *Russula nigricans* and another *Tomentella* sp. had high laccase activity (Buee et al. 2007). But when *Lactarius quietus* was grown in dead woody debris it produced mainly chitinases and less cellobiohydrolases (Buee et al. 2007). EM fungi can use N directly from litter patches added to the soil (Peres-Moreno and Read 2000), from soil organic matter (Näsholm et al. 1998), as well as NH_4^+ and NO_3^- resulting from mineralization (Finlay et al. 1992). A schematic overview of the available paths for EM fungi to access the litter-derived N is given in Figure 2.1.

EM fungi improve overall N uptake by increasing nutrient absorbing surface due to an extensive external mycelia (Bending and Read 1995). External hyphae can absorb less mobile forms of nutrients beyond the depletion zones that develop around the roots, and additionally, due their fine structure, the hyphae can scavenge nutrients in root-inaccessible soil microsites (Finlay and Read 1986, Read 1991, Finlay et al. 1998, Tuomi et al. 2001, Gobert and Plassard 2008, Alvarez et al. 2009). External mycelia differ greatly among species. Based on structure, abundance and lengths of external mycelia EM fungi have been classified as contact, short-distance, medium-distance, and

long-distance exploration types (Agerer 2001). Contact exploration type EM posses a smooth mantle with no or very few emanating hyphae. Short-distance exploration types have usually short, but dense emanating hyphae. Medium-distance exploration type may form rhizomorphs and the hyphae are more extended in the surrounding soil than those of short-distance exploration types. Long-distance EM exploration types are characterized by vessel-like hyphal structures called rhizomorphs capable of nutrient transport over long distances (Agerer 2001).

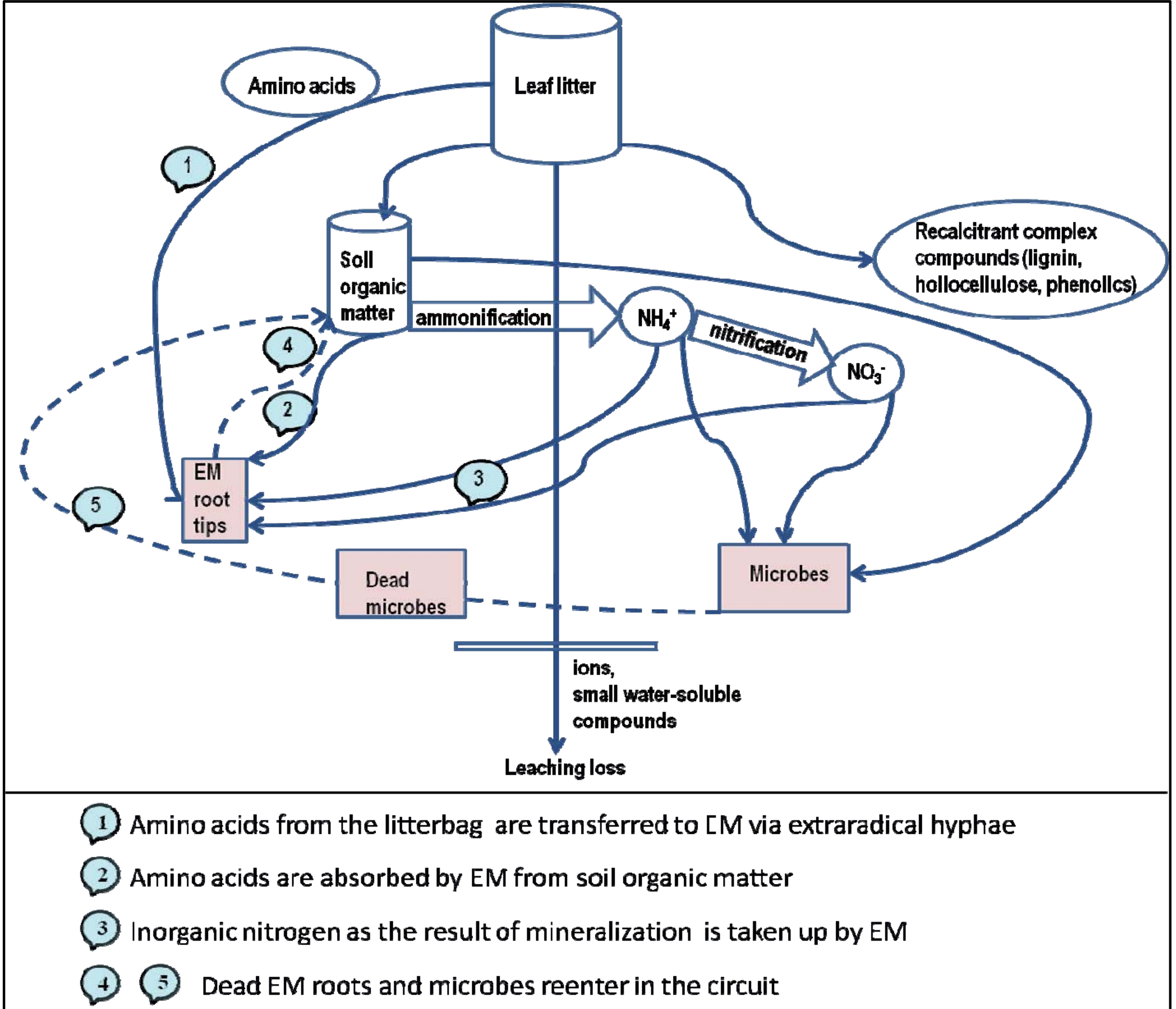

Figure 2.1: Schematic overview of different paths of litter-derived nitrogen to ectomycorrhizal root tips

It has been shown than N originating from beech litter appears in EM roots and leaves of mature trees after six and nine months, respectively (Zeller et al. 2000). However, there is no information about EM fungal species involved in the N transfer from litter to root. Therefore, it is unclear if functional diversity exists amongst EM fungal species for accumulation of litter-derived N in colonized root tips.

In the present study, the accessibility of litter-derived N for the most common EM fungal species in a beech forest was investigated over a time scale of 18 months.

The following hypotheses were tested:

- ^{15}N mobilization by EM fungi differs among fungal species in the first phase of liter decomposition (six to nine months). Long distance EM exploration types accumulate more labeled N than short distance ones, because of their higher accessibility to the litterbags.
- Differences in ^{15}N accumulation between EM fungal species decrease over time with the increasing availability of litter-released N via the soil.

To test these hypotheses mesh bags filled with ^{15}N labeled beech leaf litter were deposited for 18 months in the top soil of an old growth beech forest. ^{15}N enrichment was regularly measured in soil, fine roots and root tips colonized by different EM fungal species. To distinguish between N uptake from litter patches and from the soil, a set of litterbags were removed after 14 months and the incorporation of the label in EM root tips and roots in the presence and absence of the litterbags was measured.

2.2. Materials and methods

2.2.1. Site description

The study site was located in a 70 to 80-year old beech stand in Tuttlingen forest, a low mountain range in southern Germany (longitude 8°45`E; latitude 47°59`N) at an altitude of 760 to 820 m above sea level. Mean annual regional air temperature was 6.6°C and the sum of annual precipitation amounted 856 mm. The soil is characterized as Rendzic Leptosol derived from limestone and marls. The organic layer comprises L and Of horizons of varying depths (0-7 cm). The soil pH was 5.6 and C/N ratio 26.7 (Dannenmann et al. 2007a). The Ah horizon had a pH value of 6.1 and a C/N ratio of 14.2. The study site is exposed to low atmospheric N deposition (<10 kg N $ha^{-1}year^{-1}$). Further details of the site, stand, and soil have been described elsewhere (Dannenmann et al. 2007a, 2007b, Holst et al. 2004).

2.2.2. Litter and Litterbags

^{15}N-labeled beech leaf litter was produced as described by Zeller et al. (1998). Leaves were collected in fall and dried at room temperature. N concentration in leaf litter was 1.134 % with 1.137 atom %

^{15}N enrichment. Litterbags (10 x 3 cm) were manufactured of nylon mesh (A. Hartenstein GmbH, Würzburg, Germany) with a mesh size of 50 µm which allows hyphae, but not roots, to penetrate. Each bag was filled with 5.00 g dry beech leaves and sealed.

2.2.3. Field study and sampling design

A rectangular plot (12.0 x 17.0 m), comprising nine trees was established on 14th April 2008 (Figure 2.2A). Soil cores of 0.03 m diameter and 0.1 m depth (70 ml) were removed at about one meter distance from the stem of a tree and used for analysis of EM root tips, soil and roots (T_0). The litterbags were inserted into the cavities created by soil removal, in tight contact with surrounding soil. The bags were numbered (1-29) and their position was marked with a flag to enable retrieval. Each sample consisted of a soil cylinder (0.08 m diameter and 0.1 m height), which contained a litter bag cavity in its center. Therefore a 2.5 cm soil ring around cavity was harvested (Figure 2.2B). Samples were collected on three further occasions 14th October 2008 (T_1), 19th June 2009 (T_2), and 8th October 2009 (T_3). At T_2, seven litterbags were removed without soil cores. At T_3 soil cores with ($T_{3(+)}$) and without litterbags ($T_{3(-)}$) were collected as described above. Unlabelled soil cores were taken from an adjacent site for determination of ^{15}N natural abundance.

2.2.4. Sampling of soil, roots and ectomycorrhizas

A total of nine at $T_{1,}$ six at T_2 and $T_{3(+)}$, and seven at $T_{3(-)}$ samples were harvested at the positions shown in figure 2A. The litterbag was removed from the soil core and aliquots of leaf litter were sampled for isotope analysis. The litter was dried at 50°C for 2 days to determine of leaf mass loss (Bärlocher 2005).

The roots were carefully removed from the soil cores and washed with deionised water. The biomass of roots in each soil core was determined. The roots (diameter ≤ 2 mm) were spread under a dissecting microscope (Stemi SV 11; Zeiss, Jena, Germany). All root tips per soil core were counted and assigned according to their morphology to one of the following three fractions: vital ectomycorrhizal (EM), vital non-ectomycorrhizal (NM) and dead (DR) root tips. Live and dead roots were distinguished according to Allen et al. (2000). The EM root tips were morphologically categorized following the procedure and identification keys of Agerer (1987-1996): color, shape, branching pattern, mantel surface texture, hyphal structure and abundance were recorded. All different morphotypes were photographed (Leica DFC 420 C, Wetzlar, Germany). EM fungi were classified according to exploration types as contact, short distance, intermediate distance, and long distance exploration type (Agerer 2001). EM fungal species sufficiently abundant for isotope

analyses were identified by internal transcribed factor (ITS) sequencing (Pena et al. 2010). The sequences have been deposited in NCBI GenBank with the GenBank accession numbers JF495172 – JF495178.

For isotope analysis, different EM species, NM and DR root tips were collected under a dissecting microscope. EM tips ensheathed by mantel were excised at the last lateral root ramification. NM tips were collected at the level of the youngest and active "white" zone (Enstone et al. 2001), DR tips of similar lengths as NM tips were used. Samples were freeze dried and weighed (without milling) in tin capsules (4 x 6 mm). One sample of root tips consisted of 0.3 – 0.7 mg dry mass. Depending on the size of the morphotypes collection of 10 and 60 root tips was required.

After morphotyping the roots were fragmented and subsamples were randomly collected for isotope analysis. The remaining roots were dried at 65°C and the dry mass was determined.

Soil samples of each soil core were collected and kept for isotopes analysis. Gravimetric soil water content (SWC) was estimated as the weight loss of oven dried samples (105°C, until constant mass).

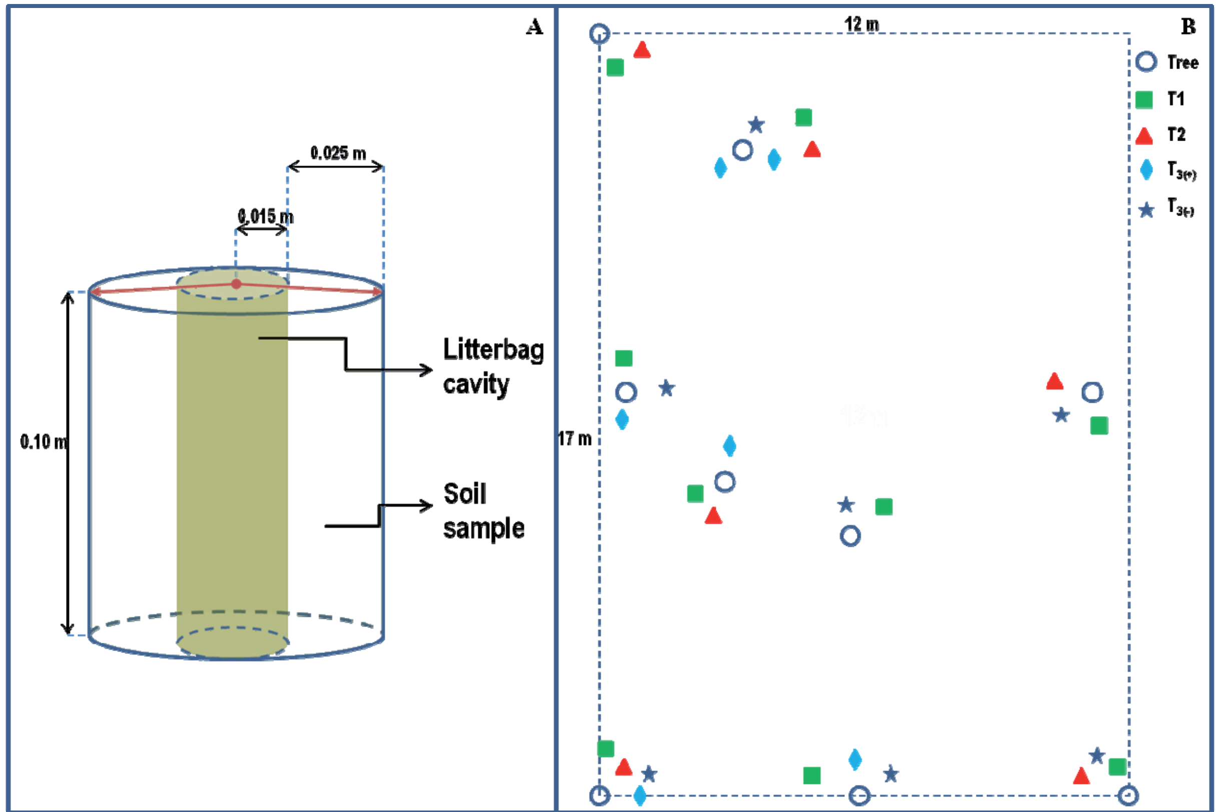

Figure 2.2: A. Image of the cavity where the litterbag as inserted and the soil sample representing a 2.5 cm soil ring around cavity. B. The experimental plot included 9 beech trees and 28 litterbags distributed at about 1 m distance from the tree. The litterbags were exposed in April 2008 and sampled at three occasions: October 2008 (T1 squares), June 2009 (T2 triangle), and October 2009 (T_{3+} diamond). T_{3-} star represent the samples harvested in two steps: first the litterbag at T2 and then the soil at T3.

2.2.5. C, N and ^{15}N analysis and calculations

For the analysis of total C, N and ^{15}N, samples of soil, roots, and litter from the mesh bags were freeze dried and milled (MM2, Retsch, Haan, Germany). Samples were weighed (S4, Sartorius, Göttingen, Germany) into tin capsules (4 x 6 mm for biological materials and 5 x 9 mm for soil, IVA Analysentechnik e.K., Meerbusch, Germany).

The measurements were made with an isotope ratio mass spectrometer (IRMS Delta ^{plus}, Thermo Finnigan Mat, Bremen, Germany) coupled to an elemental analyzer (EA 1108, Fisons, Rodano, Italy).

The N isotopic composition was expressed as $\delta^{15}N$ (‰) against the standard of atmospheric nitrogen (air):

$$\delta\,^{15}N\,(‰) = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000$$

where R is the ratio ^{15}N to ^{14}N isotope, in the sample and in the standard, respectively.

The amount of ^{15}N in the leaf litter and roots was calculated as follows:

$$^{15}N\ \text{content}\ (\mu g) = [\text{biomass (g) x N concentration x }^{15}N\ \text{atom \% excess}] / 100$$

where the biomass represents the dry mass of the leaf litter in litterbag, or that of the all fine roots found into a sample,

$$^{15}N\ \text{Atom \% excess} = \text{Atom \%}_{labeled} - \text{Atom \%}_{unlabelled}$$

$$\text{Atom \%} = 100 \text{ x } [R_{sample} / (1+ R_{sample})]$$

where labeled and unlabelled refers to samples affected by application of ^{15}N tracer and samples that contain only natural concentrations of N isotopes, respectively.

Relative incorporation of ^{15}N tracer in roots was calculated as follows:

$$\text{Relative incorporation (\%)} = [^{15}N\ \text{content}_{roots} / (^{15}N\ \text{content}_{leaf\ litter\ at\ T0} - {}^{15}N\ \text{content}_{leaf\ litter\ at\ T})] \text{ x } 100$$

where T is the sampling time.

2.2.6. Statistical analysis

Statistical analysis was performed using Statgraphics Plus 3.0 (StatPoint, Inc., St Louis, MO, USA). When necessary, data were transformed (logarithms or square root) to satisfy the criteria of normal distribution and homogeneity of variance. When data transformation did not meet these requirements, the Kruskal-Wallis and Mann-Whitney W tests were applied instead of analysis of variance (ANOVA). Means or medians were considered to be significantly different from each other, if $P \leq 0.05$.

2.3. Results

2.3.1. Root demography and EM colonization

The total number of root tips in the top soil of 10 cm depth did not vary between the four sampling dates. Usually the vital fraction amounted about 35 to 40% of the root tips. A significantly higher fraction was found at T1 (Table 2.1). NM root tips were seldom, usually less than 1% of all tips. A total of fourteen different EM species were observed based on morphological differences, but only seven of them occurred at each of the four sampling dates: UEM *Humaria* and *Cenococcum geophilum* (Ascomycota), *Boletus pruinatus*, UEM *Cortinarius*, *Tomentella badia*, UEM *Sebacina* and *Russula cuprea* (Agaricomycetes, Basidiomycota). *R. cuprea* is a contact exploration type. *H. hemisphaerica*, *C.geophilum*, and UEM *Sebacina* are short-distance, *T. badia* short to medium-distance, UEM *Cortinarius* medium-distance and *B. pruinatus* long-distance exploration types, respectively (Supplement S1). *C. geophilum* was the most frequent fungal species, colonizing between 26 and 47% of the root tips at different samplings date (Table 2.1).

Table 2.1: Relative abundance of EM fungal species. Vitality Index represents the percentage of vital root tips. No. of root tips represent the total number of root tips found in a soil core of 1000 ml. Mean values ± SE ($6 \leq n \leq 9$). Different letters indicate significant differences at $P \leq 0.05$

	UEM *Humaria*	C. geophilum	*B. pruinatus*	UEM *Cortinarius*	*T. badia*	UEM *Sebacina*	*R. cuprea*	*T. viridula*	UEM *Tomentella*	MT 7	MT 12	MT 13	MT 14	MT 15	Vitality Index (%)	No. of root tips
April 08 [T_0]	9.6 ± 3.0	47.0 ± 2.6	3.3 ± 1.4	9.6 ± 2.5	5.1 ± 1.7	15.4 ± 5.1	2.9 ± 1.3	1.5 ± 1.2	0.0 ± 0.0	5.2 ± 3.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	32.5 ± 2.7^{a}	3573 ± 794^{a}
October 08 [T_1]	16.5 ± 2.3	35.4 ± 3.8	12.1 ± 3.5	14.4 ± 6.6	16. ± 3.5	3.5 ± 1.8	1.6 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	70.2 ± 3.0^{c}	2991 ± 181^{a}
June 09 [T_2]	6.8 ± 3.0	41.8 ± 6.0	3.6 ± 1.9	6.1 ± 5.0	7.5 ± 1.9	17.1 ± 9.1	3.6 ± 1.9	0.0 ± 0.0	13.3 ± 6.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	34.0 ± 5.4^{ab}	3966 ± 591^{a}
October 09 [$T_{3(+)}$]	1.2 ± 0.8	41.1 ± 9.6	1.9 ± 1.9	6.2 ± 4.5	3.5 ± 1.5	1.5 ± 1.5	2.1 ± 1.2	9.7 ± 2.9	5.9 ± 3.9	22.6 ± 6.6	0.4 ± 0.0	0.1 ± 0.1	4.4 ± 1.2	0.1 ± 0.1	35.6 ± 5.2^{ab}	3645 ± 323^{a}
October 09 [$T_{3(-)}$]	0.8 ± 0.7	25.6 ± 7.2	0.8 ± 0.5	19.8 ± 10.6	4.9 ± 2.9	2.6 ± 2.4	1.9 ± 0.9	5.8 ± 2.8	7.1 ± 4.0	13.3 ± 6.0	1.4 ± 1.1	1.9 ± 0.0	14.4 ± 5.4	0.0 ± 0.0	46.1 ± 4.0^{b}	3845 ± 384^{a}

2.3.2. N and ^{15}N dynamics in decomposing litter

Six months after the deposition of the mesh bags, the litter mass loss was in the range of 20% and after 18 months about 46.5% (Figure 2.3A).

Similar with other studies (Staaf 1980, Zeller et al. 2000, Zeller et al. 2001), there was an about 10% increase in the N concentration in beech leaf litter during the first six months of exposure ($\delta^{15}N$ = 0.96 ± 0.12 µg g^{-1}, P = 0.018, Figure 2.3B). Overall, N concentration was relatively stable during the time course of 18 months. Therefore, the mass loss of the litter in

the mesh bag resulted in a corresponding loss (-56%) in the total amount of N from 56.7 ± 0.9 to 32.2 ± 4.1 mg. $\delta^{15}N$ values of leaf litter decreased moderately by about 10%, probably caused by initial incorporation of external N (Figure 2.3C).

C to N ratio in the litter did not vary significantly during the exposure, and amounted about 40.8 ± 0.9 (Figure 2.3D)

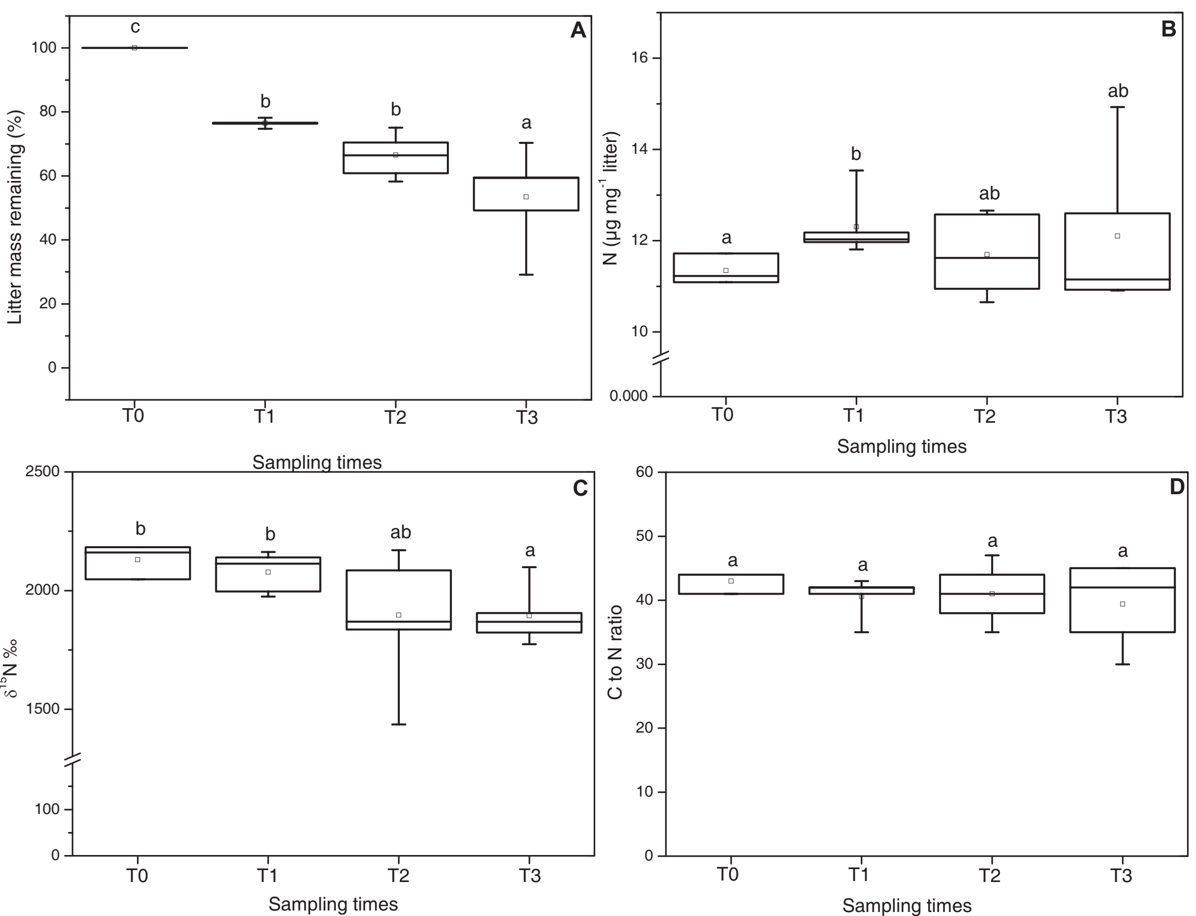

Figure 2.3: Temporal variation in litter mass loss (A), nitrogen concentrations (B), $\delta^{15}N$ values (C) and C:N ratio (D) of leaf litter deposited in the field. Box plots represent $5 \leq n \geq 7$. Different letters indicate significant differences at $P \leq 0.05$ (Mann Whitney-W test).

2.3.3. ^{15}N tracer in soil and roots

The natural abundance $\delta^{15}N$ in the soil of 0-10 cm depth was similar to that found in other studies (Zeller et al. 2000, Stoelken et al. 2010) with values ranging between -1.094 and 0.790 ‰ (Table 2.2). In the soil, ^{15}N released from litterbags resulted in significantly higher $\delta^{15}N$ values (P = 0.014) after 18 months of exposure (Table 2.2). $\delta^{15}N$ values in soil increased with litter decomposition time (Rs = 0.65, P = 0.008). The removal of litterbag significantly affected the soil $\delta^{15}N$ values (P = 0.020), which were about two times lower in absence of labeled litter than in their presence. Soil water content did not vary significantly between sampling times, ranging from 27 to 34%.

The ^{15}N tracer originating from labeled litter was detected in fine roots as early as 14 months after exposure (Table 2). $\delta^{15}N$ values in roots became greater over the time of litter decomposition (Rs = 0.65, P = 0.002). Similar as in soil, the absence of litterbag resulted in about 5 folder lower $\delta^{15}N$ values in the fine roots.

Table 2.2: Temporal variation in $\delta^{15}N$ measured in the soil (0-10 cm depth) and fine roots. Median values; in brackets: minimum and maximum value ($4 \leq n \leq 6$). Different letters indicate significant differences at $P \leq 0.05$.

	Sampling time	$\delta^{15}N$ ‰
Soil		
	T_0	0.038^a (-1.094, 0.790)
	T_1	1.198^a (0.354, 1.716)
	T_2	-0.039^a (-0742. 4.647)
	T_3	7.768^c (5.488, 16.809)
	$T_{3(-)}$	4.310^b (3.100, 6.917)
Fine roots		
	T_0	-1.399^a (-3.292, 1.754)
	T_1	1.760^a (-0.271, 3.669)
	T_2	2.540^{ab} (-0.609, 14.795)
	T_3	18.202^c (11.984, 87.445)
	$T_{3(-)}$	7.252^b (4.677, 14.921)

About 0.08 % of the ^{15}N released from labeled leaf litter had been incorporated into the root biomass after 18 months of litter degradation (Table 2.3). A similar fraction was also found in roots after six

months of litter exposure, due the massive root biomass at that sampling time point (Table 2.3). During the 4 months following removal of the litterbag, the fraction of ^{15}N incorporated in fine roots did not significantly change (Table 2.3).

Table 2.3: Relative incorporation of ^{15}N released from leaf litter in the fine root biomass. Mean values ($4 \leq n \leq 6$). Different letters indicate significant differences at $P \leq 0.05$.

Sampling time	^{15}N released from leaf litter (µg per litterbag)	Root biomass in 430 ml soil (g)	Incorporation of ^{15}N in fine roots (%)
T_1	119 ± 15	0.53 ± 0.10	0.102 ± 0.030**b**
T_2	236 ± 14	0.35 ± 0.06	0.027 ± 0.012**a**
T_3	307 ± 41	0.15 ± 0.01	0.082 ± 0.025**b**
$T_{3(-)}$	236 ± 14	0.18 ± 0.00	0.034 ± 0.015**ab**

The EM root tips colonized by different fungal species differed in natural ^{15}N enrichment measured at T_0, before deposition of labeled leaf litter. Except T_3 sampling, 18 months after litter exposure, all vital EM root tips were enriched in ^{15}N compared with the fraction of dead root tips (Figure 2.4H). The strongest ^{15}N enrichment was found in UEM *Cortinariaceae* (Figure 2.4G).

Six months after exposure to ^{15}N labeled litter, ^{15}N enrichment increased significantly in all categories of root tips, except those colonized by *B. pruinatus* (Figure 2.4).

After 14 months of labeled litter exposure, the root tips colonized by UEM *Cortinariaceae*, *T. badia* and *R. cuprea* displayed higher ^{15}N enrichments than those colonized by other species (Figure 2.4).

All EM root tips showed a strong increment in $\delta^{15}N$ after 18 months of labeled litter exposure. The pattern of the ^{15}N enrichment in the dead root tips followed the one found in EM root tips. However, it was always lower than in vital root tips.

The exclusion of the paths of the direct uptake of ^{15}N via extraradical hyphae by removal of the litter bags (Figure 2.1) induced significant effects on ^{15}N accumulation in the root tips colonized by different EM fungi. For example, the root tips colonized by *T. badia* showed a 5 times higher enrichment in the presence than in the absence of litterbag (Figure 2.4B).

Root tips colonized by different EM fungi revealed specific patterns of $\delta^{15}N$ enrichment, during the time course of the experiment. UEM *Humaria* and *C. geophilum* showed initially a significant increment of $\delta^{15}N$ values in the root tips after six months of litter exposure, but not further accumulation. After 18 months the $\delta^{15}N$ was further increased in the root tips colonized by these two species, but there were not differences in presence or absence of the litterbag (Figure 2.4).

After six month of tracer presence in the field, *B. pruinatus* did not yet show a significant enrichment (P = 0.074). Only after one year, the $\delta^{15}N$ values increased significantly in comparison with the natural abundance in this EM fungal species (Figure 2.4).

The root tips colonized by UEM *Sebacina* showed a significant increment in $\delta^{15}N$ after 6 months after litterbag deposition, but afterwards the ^{15}N values remained almost constant (P = 0.056).

T. badia was the EM fungus which displayed the most intense accumulation of ^{15}N at all sampling occasions (Figure 2.4B), while *R. cuprea* had the lowest $\delta^{15}N$ values (Figure 2.4F).

2.4. Discussion

2.4.1. The fate of ^{15}N released from litter

Our study shows a clear hierarchy of tracer accumulation, first in EM, then in roots and finally in soil. In each compartment, the tracer accumulation at any time is the net result of ^{15}N influx and export to other pools. In line with other studies (Zeller et al. 2000, Zeller et al. 2001) we found that during 18 months after exposure in the field, about 46 % of the introduced labeled litter mass was lost. As N release from decomposing labeled litter occurred at similar rates with litter mass loss, it was a direct effect of leaching of soluble compounds (Berg and McClaugherty, 2008). We also found an accumulation of N in the litter, which must have been caused by exogenous N deposition from throughfall, and from surrounding soil due to microbial transfer (Berg and McClaugherty, 2008). The input of exogenous N did not exceed the release of litter N during the time course of the experiment, since the experimental site was exposed to low N deposition of <10 kg N ha^{-1} y^{-1} (Dannenmann et al. 2008). Earlier studies showed a net loss of N only after two years of litter exposure (Staaf 1981, Zeller et al 2001). ^{15}N tracer was detected in the soil (0-10 cm depth) after 18 months, while other authors (Zeller et al. 2000, Zeller et al. 2001) have found higher $\delta^{15}N$ values in the upper soil (2 cm depth), and no alteration of $\delta^{15}N$ in the deeper soil (8 cm depth) in the first year after labeled litter deposition. In contrast with our experimental design, in the above cited studies the litterbag was placed on the F organic layer and not inserted in the top soil.

The detection of ^{15}N tracer in the soil in the absence of litterbag, when $\delta^{15}N$ values were close to natural abundance at the time of labeled litter removal, may be explained by ^{15}N input of labeled necromass. Although a higher contribution to soil ^{15}N pool was expected from the turnover of enriched EM root tips, $\delta^{15}N$ values in dead root tips were negative until 14 months after tracer application.

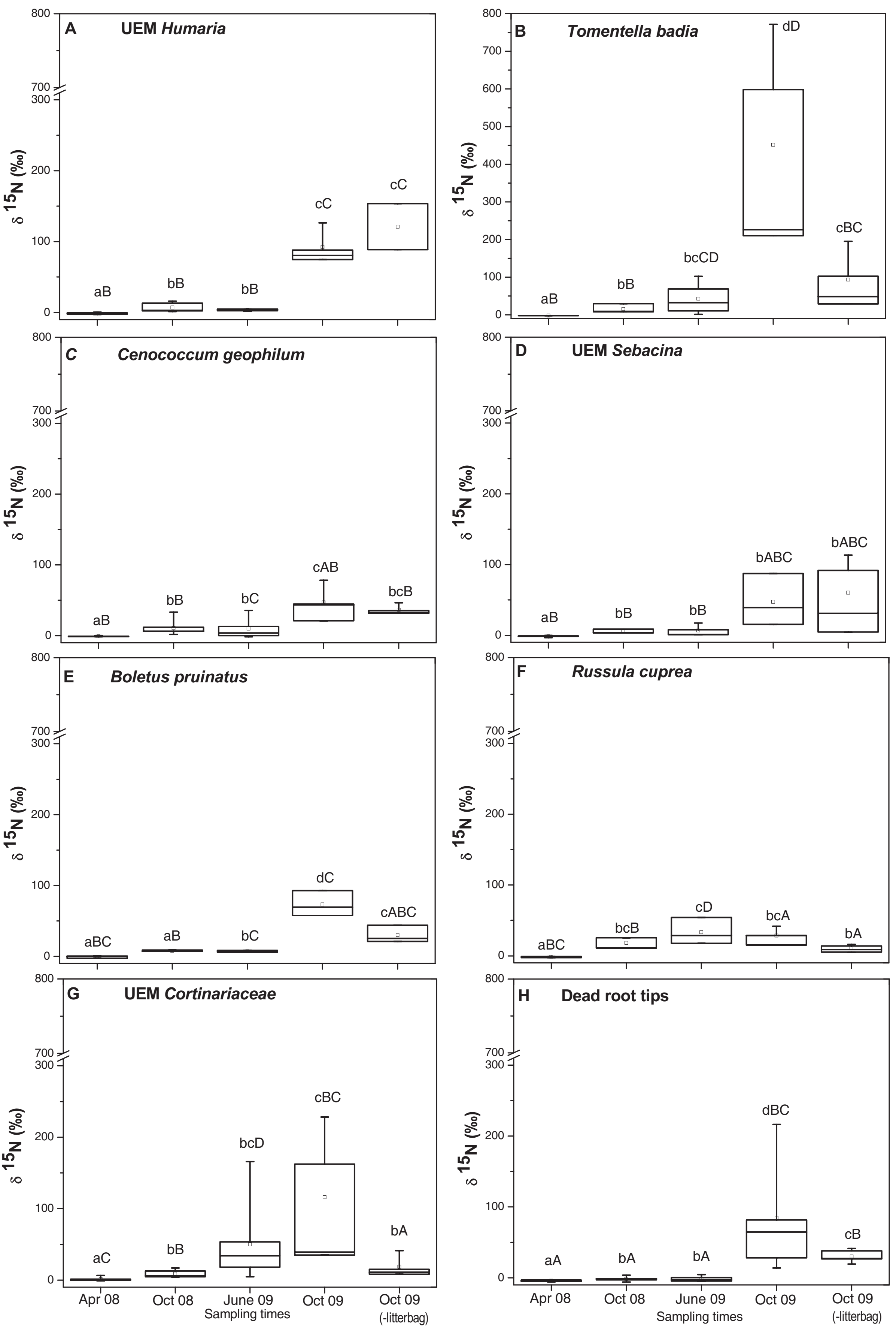
A UEM *Humaria*
B *Tomentella badia*
C *Cenococcum geophilum*
D UEM *Sebacina*
E *Boletus pruinatus*
F *Russula cuprea*
G UEM *Cortinariaceae*
H Dead root tips
δ 15N (‰)
Apr 08
Oct 08
June 09
Oct 09
Oct 09 (-litterbag)
Sampling times

Figure 2.4: Temporal variation of $\delta^{15}N$ values of root tips colonized by different EM fungi and of dead root tips. Box plots represent $2 \leq n \leq 8$ (2 replicates were measured for *Boletus pruinatus*, UEM *Sebacina* and *Russula cuprea* at T_1 and UEM *Humaria* at $T_{3(-)}$). Different lower case letters indicate significant differences at $P \leq 0.05$ (Mann Whitney- W test) between sampling dates while different capital latters indicate significant differences between $\delta^{15}N$ values of root tips colonized by different EM fungi and of dead root tips at each sampling time.

The amount of ^{15}N incorporated in the fine roots after six months of litter exposure exceeded the values found after 14 months of exposure because of the unusually increased root biomass. Six months after introduction of the litterbag, the root biomass and the vitality index were higher than those found during the following time of the experiment. The soil relative water content was approximately constant at different sampling times. Therefore, an influence of water availability on root tips vitality was excluded. By construction of soil cavities that hosted the litterbags, the roots were severed and probably local soil chemical (i.e. water and nutrient availability) and physical (i.e. bulk density) alterations occurred. The soil disturbance was probably similar to that induced by installation of minirhizotons, which caused an immediate root proliferation (Joslin and Wolfe 1999). A fine root production rate higher than the mortality rate was also reported for the first growing season following soil disturbance (Satomura et al. 2007).

EM revealed a high capacity to acquire litter-derived N. The $\delta^{15}N$ values were significantly higher after six month of labeled litter exposure and increased continuously over the time. Even if EM fungi accessed litter-derived organic N, or N fractions mineralized in the mean time, they are probably strong competitors with soil microbes, since the tracer was detected much later in the soil.

2.4.2. Functional diversity of EM with respect to N accumulation

The structure of the EM community found in the vicinity of the litterbags was typical for temperate beech forest, dominated by *Tomentellaceae*, *Russulaceae*, *Cortinariaceae*, and *Boletales* (Buee et al. 2005, Buee et al. 2007, Courty et al. 2008). EM fungal species composition fluctuated between sampling dates, as reported also by other authors (Courty et al. 2008, Pena et al 2010). The most frequent species was *C. geophilum*, a short exploration type among EM fungi. However, in the community all four main EM exploration types were represented.

EM fungi may take up N directly from the litterbag taking the advantage of external mycelia (path 1 in figure 1, Lindahl et al. 2002), or from the soil (paths 2 and 3 in figure 1, Knops 2002, Chapman 2006). In the first year after litter deposition ^{15}N enrichment increased significantly in EM root tips, while the tracer was not detected in the soil. We hypothesized that EM fungi belonging to medium or long exploration types may provide an advantage by acquiring more N than other EM species. Based

on our data from the first sampling we have to reject this hypothesis. Six month after litter deposition, all EM species displayed similar ^{15}N enrichments, except *B. pruinatus*, although this was the only species with long exploration type mycelia. Perhaps, the external mycelia outreached the tracer area or the ^{15}N accumulation was diluted due the high mycelial biomass.

EM fungi vary in their natural abundance of N isotopes (Hoegberg 1997**,** Gebauer and Taylor 1999, Lilleskov et al 2002, Trudell et al. 2004, Taylor et al. 2003). Trudell et al. (2004) have suggested that differences of isotope ratios are the effect of interaction of multiple ecophysiological factors such as organic N use, metabolic processing, and the proportion of N transferred to the plant partner or mycelia morphology. The comparison of isotope ratios of EM fungal sporocarps collected in the field showed a strong positive relationship between $\delta^{15}N$ values and the use of organic N (Lilleskov et al 2002). The metabolic processes that contribute to N isotopic fractionation in fungi are conserved across the taxa (Henn and Chapela 2004). Hobbie and Agerer (2010) suggested that EM taxa with larger external mycelia should have higher $\delta^{15}N$ values in sporocarps, because of larger N accumulation. In contrast to this suggestion, in our study, $\delta^{15}N$ natural abundance of the EM root tips did not vary among fungal species belonging to different exploration types, except the root tips colonized by UEM *Cortinariaceae*. These EM displayed significantly higher values. *Cortinarius* is a fungus with medium distance exploration type of high biomass and it has the capacity to use organic N (Lilleskov et al. 2002, Treseder et al. 2008).

Root tips colonized by the two ascomycetes, UEM *Humaria* and *C. geophilum*, showed similar $\delta^{15}N$ enrichment patterns over the time. They displayed no differences in $\delta^{15}N$ from 6 to 14 months of labeled litter exposure, while 4 month later, concomitant with detection of the tracer in the soil, the enrichment increased significantly, suggesting a higher accessibility to soil N. In the same time, the interruption of EM hyphal access to labeled litter by removing the litterbag had no effect on $\delta^{15}N$ in the root tips colonized by UEM *Humaria* and *C. geophilum*. These two ascomycetes have been described as short-distance exploration types with emanating hyphae that have a limited growth into the surrounding soil (Agerer 2001). They are thus obligated to scavenging for nutrients in their vicinity *C. geophilum* is a ubiquitous fungus (Tedersoo et al. 2003), which has been detected more frequently in forest soils with higher than in those with lower levels of organic matter (Cullings et al. 2001). Abuzinadah and Read (1996) found in controlled studies that *C. geophilum* was more productive when peptides and proteins than ammonium were the N source. Although *C. geophilum* has the capacity to access organic N, its effectiveness for litter-derived N was lower that of other fungi.

Other fungi which were classified as short distance and contact exploration types, respectively, were UEM *Sebacina* and *Russula cuprea*. They revealed a behavior similar to that found for UEM *Humaria* and *C. geophilum*, regarding the effect of litterbag removal on ^{15}N accumulation. However, the root tips colonized by *R. cupraea* displayed little $\delta^{15}N$ enrichment in comparison with other fungi, except after 14 months of litter exposure. *R. cuprea* has been described as a "protein fungus" and has the capacity to mobilize organic N directly from proteins (Lilleskov et al 2002). The lack of external hyphae forces it to access only its immediate vicinity. *R. cupraea* was a strong accumulator for ^{15}N, because at that time point the label in the soil was still not detectable.

The removal of the labeled litterbags from the field influenced the $\delta^{15}N$ enrichment of the EM root tips of medium or long distance exploration types. These fungi, UEM *Cortinarius B. pruinatus*, and *T. badia*, form external mycelia with hyphae and rhizomorphs that may penetrate massively into the litterbag. UEM *Cortinarius* revealed low $\delta^{15}N$ values in the absence of labeled litter. Before litterbag removal *Cortinarius* revealed the highest ^{15}N acquisition among EM fungi. *Cortinarius* fungi live typically in organic soil and use protein and amino acids as N source (Lilleskov et al. 2002, Cullings et al. 2003, Tresender et al. 2008). Brandrud and Timmermann (1998) have explained the dramatic decline of *Cortinarius* in response to increased forest nitrogen deposition by its adaptation to N-limited environments, possessing a very efficient and complete uptake of all available N forms. The data from our study suggest that *Cortinarius* has a clear preference for direct N capture from degrading leaf litter. *T. badia* ectomycorrhizas have been classified as short to medium distance exploration types (Agerer 2003). *T. badia* revealed the highest level of ^{15}N enrichment in our study and was strongly responsive to litterbag removal. This demonstrates its access to N in the litterbag. Tomenteloid ectomycorrhiza have been previously described as colonizers of wood debris (Tedersoo et al. 2003), having a high capacity to produce enzymes involved in litter degradation (Koljalg et al. 2000). *T. badia* has a strong chitinase activity (Buee et al. 2005), which may cause N mobilization from soil necromass, which may be an important supply route when the litter was lacking. *B. pruinatus* is a long-distance exploration type. It forms rhizomorphs optimized for long distance transport of solutes and its external mycelia can be found up to 10 m away from the colonized root tips (Kjoller 2006). As expected, the removal of litterbags induced diminished tracer acquisition as an effect of cessation of direct N mobilization via hyphae. Nonetheless, in contrast with *Cortinarius*, ^{15}N was still accumulated in *B. pruinatus* at significantly higher rates than before litterbag withdrawal. *Boletus* sp. have higher chitinase activity compared with other EM fungi (Courty et al. 2005). Therefore, in the absence of litterbag *B. pruinatus* may have had the capacity to mobilize N by degrading chitin of fungal walls of dead ^{15}N labeled microorganisms in the soil.

EM fungi accessed litter-derived N already during the first six months of litter deposition. EM fungal species with extensive external mycelia, but also with abilities to take up organic N, showed a better gain of litter-derived N. An exception was *B. pruinatus* that showed moderate enrichments, despite the fact that it may form rhizomorphs. According with the theory of functional complementarity (Koide 2000, Buee et al 2007), EM fungal communities consist of species with different abilities: *B pruinatus* may have higher competence for water transport (Duddridge et al. 1980, Plamboeck et al. 2007) than for N uptake. Overall, the advantage of well developed external mycelia for litter derived N acquisition did not appear in the first phase of litter decomposition, but only later on. Probably, in the first months of litter deposition, when the fraction of soluble compounds was leached, the EM fungi competed for tracer in their immediate vicinity and only at a later stage they invaded the litterbag and obtained ^{15}N via external mycelia.

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Supplement 2.1

Morphological characterization of EM morphotypes formed with roots of mature beech. For morphotypes identified by ITS sequencing the GenBank accession number, data of best alignment by BLAST in UNITE (accession numbers starting with UDB) or NCBI (other accession numbers) are indicated. Bar 500 µm.

Identification	Best BLAST match(es) (% similarity)	Description	
Boletus pruinatus JF495173	UDB000018 *Xerocomus pruinatus* (99%) FM995548 Uncultured *Boletaceae* (99%)	Monopodial ramified, brown or dark green silvery-white, rhizomorphs present. Long-distance exploration type	
Cenococcum geophilum FJ403485	UDB002301 *Cenococcum geophilum* (98%) EU668240 Uncultured *Cenococcum* (99%)	Single or rarely dichotomously branched, black, compact mantle, black rigid hyphae emanating from mantle Short-distance exploration type	

Russula cuprea JF495176	UDB002420 *Russula cuprea* (98%) AY061667 *Russula cuprea* (99%)	Monopodial ramified, brown yellowish, few whitish emanating hyphae. Rhizomorphs absent Contact exploration type	
Tomentella badia JF495174	UDB000952 *Tomentella badia* (99%) HM748639 Uncultured *Tomentella* (99%)	Monopodial-dichotomously branched, brown, with only few emanating hyphae. Short distance exploration type	
Tonentella viridula JF495177	UDB000261 *Tomentella viridula* (99%) AF272914 *Tomentella viridula* (99%)	Monopodial dichotomous branched, brown, with yellow tips, with short, regular emanating hyphae. Short-medium distance exploration type	
UEM *Cortinarius* FJ823395	UDB000068 *Cortinarius sertipes* (96%) HQ204640 Uncultured *Cortinarius* (99%)	Irregularly pinnate ramified, brown-reddish, with abundant white emanating hyphae. Medium-distance exploration type	
UEM *Humaria* JF495172	UDB000988 *Humaria hemisphaerica* (94%) EU024887 Humaria (99%)	Single or monopodial branched, light brown, rhizomorphs absent Short-distance exploration type	

UEM *Sebacina* JF495175	UDB000975 *Sebacina epigaea* (97%) AY296259 Sebacinaceae (98%)	Monopodial ramified, brown yellowish, with white, long emanating hyphae Short-distance exploration type	
UEM *Tomentella* JF495178	UDB003320 *Tomentella subclavigera* (98%) AM181395 Uncultured *Thelephoraceae* (99%)	Monopodial-dichotomously branched, dark brown, rhizomorphs absent, wrinkled mantle surface. Short distance exploration type	
MT 7		Monopodial ramified, brown yellowish, with white, long, abundant emanating hyphae.	
MT 11		Single, unramified, brown, compact mantle, few hyphae emanating from mantle	
MT 12		Dichotomous branched, brown-grey-silver, fleeced mantle, without long mantle, without long emanating hyphae.	
MT 14		Monopodial ramified, brown reddish, with few, long, abundant emanating hyphae.	

CHAPTER 3: Nitrogen turnover in beech (*Fagus sylvatica*) root tips colonized by different ectomycorrhizal fungi under two levels of irradiance and water availability

3.1. Introduction

Nitrogen (N) is the most extensively taken up element by higher plants from the soil. In temperate forests, where under natural conditions the majority of trees have ectomycorrhizal (EM) roots (Smith and Read 2008), N is a major limiting nutrient for plant growth (Rennenberg et al. 1998, LeBauer and Treseder 2008). EM fungi are important for mineral N uptake since their extraradical external mycelia act as a continuation of the nutrient absorbing root surface (Tuomi et al. 2001). External hyphae can absorb less mobile forms of nutrients beyond the depletion zone that develop around the roots, and additionally, due their fine structure, the hyphae can explore root-inaccessible soil micro sites (Finlay and Read 1986, Read 1991, Finlay et al. 1998, Tuomi et al. 2001, Gober and Plassard 2008, Alvarez et al. 2009). In some cases, emanating hyphae may form vessel-like structures called rhizomorphs that are capable of nutrient and water transport along long distances (Duddridge et al. 1980, Agerer 2001). The contribution of EM associations to plant N nutrition was estimated by isotopic measurements to account for up to 80% of total N uptake (Hobbie and Hobbie 2006).
A characteristic of EM fungal communities is their high diversity (Horton and Bruns 2001, Dahlberg 2001). Plant roots are simultaneously colonized by a large number of EM fungal species (Trappe 1962, Gardes and Burns 1996, Johnson et al. 1997, Gehring et al. 1998, Rinaldi et al. 2008, Feddermann et al. 2010). For example, in a single stand of mature beech trees about 60 to 90 EM fungal species were found (Buee et al. 2005, Pena et al. 2010). The basis of this diversity and its consequences for the functions accomplished by EM communities remain poorly explored (Horton and Bruns 2001, Dahlberg 2001). Bruns (1995) has suggested that niche differentiation is a prerequisite of EM fungal richness. EM fungal species have individual abilities to exploit soil resources (Abuzinadah and Read 1989a, Abuzinadah and Read 1989b; Dighton et al. 1990; Johnson et al. 1997; Finlay 2008; Baxter and Dighton 2001, Tedersoo et al. 2003). Buee et al. (2007) proposed that the concept of functional complementarily, meaning that roots are colonized by fungal species with different, complementary functional capabilities, previously described for endomycorrhizal fungi (Koide 2000), should also be applicable to EM fungal communities. They found that different EM fungal species that colonize the same ecological niches reveal different enzymatic activities and that these activities change in different environments, i.e. niches (Buee et al. 2007). For example, in soil organic layer *Lactarius quietus* and *Tomentella* sp. expressed strong

cellobiohydrolase activities, while *Russula nigricans* and another *Tomentella* sp. had high laccase activity (Buee et al. 2007). However, when Lactarius *quietus* was grown in dead woody debris it produced mainly chitinases and less cellobiohydrolases (Buee et al. 2007). The hypothesis of functional complementarity received further support by the observations that the N contents of pine seedlings inoculated only with *Laccaria laccata* were significantly lower than those of seedlings inoculated with three fungi *Rhizopogon rubescens*, *Rhizopogon luteolus* and *Laccaria laccata* (Cho-Chou and Grace 1985).

Indirect evidence relates functional diversity of EM fungi to morphological diversity, mainly to the structure of external mycelia (Agerer 2001, Hobbie and Agerer 2010). External mycelia differ greatly among EM fungal species (Agerer 2001). Based on characteristics such as structure, abundance and lengths of external mycelia, Agerer (2001) has classified EM fungi into four main exploration types: contact, short-distance, medium-distance, and long-distance type, respectively. Contact exploration type EM possesses a smooth mantel with no or very few emanating hyphae; rhizomorphs are absent. Short-distance exploration type EM has usually short, but dense emanating hyphae; rhizomorphs are lacking. Fungal species characterized as medium-distance exploration type form rhizomorphs and the hyphae are more extended in the surrounding soil than those of the short-distance exploration type. Long-distance type EM is characterized by a smooth mantle with highly differentiated rhizomorphs. EM fungi that form rhizomorphs improve plant water relations (Duddridge et al 1980).

When soil water availability is limited, the performance of different EM species differs (Theodorou 1978, Coleman et al. 1989, Dosskey et al. 1991, Kennedy and Peay 2007, Kennedy et al. 2007), and EM fungal communities are modified (Gehring et al. 1998, Shi et al.2002). EM fungal species display different abilities to survive in a drying soil (di Pietro et al. 2007).

Differences in EM fungal communities may further result from differences in plant physiology (Gardes and Bruns 1996; Horton and Bruns 1998). EM symbiosis is dependent on the capacity of the plant to supply C to its associated fungi (Smith and Read 2008). In EM plants, about 15-30 % of recent assimilates are rapidly translocated to fungal symbionts (Finlay and Soderstrom 1992, Heinosalo et al. 2004, Hobbie and Hobbie 2006). When the plant-assimilated C allocation to roots was interrupted by girdling, the soil microbial respiration decreased in two weeks by 56%, of which 41% was due to the loss of EM mycelia (Högberg et al. 2001, Högberg et al. 2002). In addition, fruiting body formation of EM fungal species ceased (Högberg et al. 2001). In a beech forest, girdling resulted in decreased EM fungal richness (Pena et al. 2010). During two years after interrupting C translocation to roots about 40 from 90 taxa disappeared. Restriction of C flux to EM

fungi by defoliation induced a decrease in fruiting body formation of EM fungal species (Kuikka et al. 2003), suppression of morphotypes with thick mantels compared to those with thin mantels (Markkola et al. 2004), but increased the number of root tips colonized by low-biomass EM that probably require less plant derived C (Saravesi et al. 2008). Reduction of photosynthesis in deep shade also caused a strong diminution of EM root colonization rate, from 74% in sun-acclimated plants to 10% in shaded plants (Druebert et al. 2009). All these studies show that an alteration of C flux to the root EM fungi affect EM fungal community and probably the physiological functions of different EM fungal species.

An important contribution to understanding the functional diversity of EM communities with respect to N nutrition is represented by the observations showing that EM fungi differ in natural abundance of N isotopes in their biomass, i.e. sporocarps and soil mycelia (Gebauer and Dietrich 1993, Högberg et al. 1999, Gebauer and Taylor 1999, Hobbie et al. 1999, Henn and Chapela 2001, Spriggs et al. 2003, Hobbie and Colpaert 2003, Hobbie and Hobbie 2006). These variations of stable N isotopes between EM species are thought to reflect different capacities of various EM fungi for acquisition of organic or mineral N (Gebauer and Taylor 1999, Hobbie et al. 1999). Recently, Hobbie and Agerer (2010) suggested that $\delta^{15}N$ values reflect N sequestration in the fungal mycelium, and therefore fungal species characterized by higher biomass, with extensive mycelia in the soil, should possess higher $\delta^{15}N$ values.

To date, studies examining physiological differences of N uptake and processing amongst EM fungi did not address the question of the role EM fungal diversity on N nutrition. Hobbie and Colpaert (2003) and Hobbie and Agerer (2010) have explained the isotopic fractionation of N uptake from the soil in the EM symbiosis by two process: in the first step, N is transferred from the initial fungal pool to two other pools, one is represented by “immobile N” forms such as chitin and other recalcitrant compounds that are required exclusively for fungal needs and the other pool represents the “metabolically active N” that is further processed; in the second step, the metabolically active N is shared between fungal mobile N and plant N. If the N source is labeled, the newest absorbed N in the EM root tip can be identified. Therefore, it is possible to calculate a proxy for N turnover in the root tip as the ratio between the new absorbed (^{15}N) to total fungal N in the root tip.

The aim of this study was to obtain evidence for functional diversity of EM fungi based on uptake and processing of N. ^{15}N to N ratio, as a proxy of N turnover in the root tip offer direct clues for distinction amongst root tips colonized by different EM fungal species, regarding fungal abilities in N processing. The performance of different EM species for N processing was also investigated in relation to their demand of current photosynthates, by reducing the plant C productivity by shade and

drought. To achieve these goals a growth chamber experiment was conducted with young beech plants, whose root systems were either colonized by typical EM fungal communities, or either NM. The plants were exposed to light and shade, respectively, and stressed by limiting water availability. Before harvest, the plants received labeled nitrogen (^{15}N) to determine N uptake. Root demography and EM fungal colonization, ^{15}N and N contents per EM and NM root tip were measured.

3.2. Materials and methods

3.2.1. Plant cultivation

Beech seedlings (*Fagus sylvatica*) were grown from germinated beech nuts (provenance: Forstsaatgutstelle Oerrel, Niedersachsen, Germany) and planted in 1 l pots filled with forest soil, in the greenhouse, under ambient conditions, with 16 h photoperiod. To avoid fungal contamination, the germinated beech seeds were treated before planting as follows: the seed coats were removed and then seeds were kept for 12 h were kept in a sterilization solution [1 l H_2O, 1 ml fungicide Proplant (Stähler, Stade, Germany), 100 mg tetracycline (Duchefa Biochemie, Haarlem, Holland)] and washed three times with tap water.

The soil was obtained from Tuttlingen forest (latitude 47°59`N, longitude 8°45`E, see Dannenmann et al. 2009 for more details), by collecting the Ah horizon (20 cm depth). Half of the soil (O) was kept in darkness at 4°C until planting. The other half (S) was autoclaved at 121°C and 0.11 MPa (HST 6x6x6, Zirbus Technology, Bad Grund, Germany) once a week three times. The pH of the sterilized and the original soil were 6.46 (±0.07) and 7.02 (±0.02), respectively.

when the development of mycorrhiza was good (ca. 30%) in O soil and lacking on plants grown in S soil, 4-months-old beech plants were transferred to containers with sand soil and into a culture chamber under controlled environmental conditions: 16 h day length; 20°C temperature; 55% relative air humidity. The soil was a homogenous mixture consisting of peat, fine (0.3 mm) and gross sand (8 mm) (1:4:5).

3.2.2. Experimental treatments

A split-split-plot design was applied with the following components: ectomycorhizal colonization represented the main-plot; irradiance was split-plot within ectomycorrhizal colonization; and drought was a split-plot within the combination of the first two plots.

The irradiance level was established by natural light (greenhouse) with additional application of photosynthetically active radiation (PAR) of 200 µmol m^{-2} s^{-1}. Shading was obtained by installing a double layer polyethylene shading net 5x5 mm mesh (Mayer, Rellingen, Germany), which reduced irradiation to 35-40 µmol PAR m^{-2} s^{-} at plant height. The irradiance level was measured using a quantum photometer Li-185B with a quantum sensor Li-190SB (LiCor INC., Lincoln, USA). Each plant was watered 7 times per day with 8 ml of a Hoagland-based nutrient solution adapted after Dyckmans (2000), containing 0.4 mM NH_4Cl, 0.05 mM $NaSO_4$, 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 $MnSO_4$, 0.005 mM $FeCl_3$, 0.15 µM $ZnCl_2$, 0.1 µM MoO_3, 0.064 µM CuCl, pH 3.9. The only nitrogen source was ammonium. Soil moisture content measured with ThetaProbe ML2X soil moisture sensor equipped with a HH2 readout unit (Delta-T Devices Ltd, Cambridge, UK) was maintained above 0.040 m^3m^{-3}.

After 2 months of acclimatization, within each of the four growth regimes, EM plants full light, EM plants shade, NM plants full light and NM plants shade, two water levels were applied: well-watered plants and plants subjected to drought stress, for which the volume of watering solution was reduced 62.5 % to 3 ml per plant. The soil moisture content decreased below 0.035 m^3m^{-3}. The drought stress lasted for 16 days. This resulted in eight growth regimes: EM plants full irradiance control, EM plants full irradiance drought, EM plants low irradiance control, EM plants low irradiance drought, NM plants-full irradiance-control; NM plants-full irradiance-drought; NM plants-low irradiance-control; NM plants-low irradiance-drought. To characterize drought responses of the seedlings, leaf predawn water potential (Ψ_{pd}) was measured using a Scholander pressure chamber (Soil-moisture Equipment Corp., Santa Barbara, CA).

^{15}N labeling was applied on three consecutive days before harvest. NH_4Cl in the nutrient solution was replaced by 2mM $^{15}NH_4Cl$ (99 atom %) (Cambridge Isotope Laboratories, Inc, Hampshire, GB). Each seedling received 1.864 mg ^{15}N during the time course of 3 days. All seedlings (control and drought) were supplied with the same amount of nutrients, thus the control plants were irrigated supplementary with 5 ml deionized water in the intervals between nutrient solution applications.

Each of the eight growth regimes was comprised of twenty-four ^{15}N labeled and three non-labeled plants.

3.2.3. Harvest

The plants were harvested after 72 h of ^{15}N labeling. Roots were carefully washed with deionised water to remove isotope adhering and separated in fine roots (<1mm in diameter) and coarse roots (>1mm in diameter). Biomass of foliage, stems and roots was determined for each tree. Aliquots of

fresh material of the different fractions were taken and freeze-dried for further isotope measurements. The remaining biomass, except the roots, was dried at 60°C and used for dry mass estimation. Roots were kept at 4°C in plastic bags until morphotyping.

3.2.4. Root analysis and EMF morphotyping.

For each growth regime, the whole root system of ten seedlings was analyzed. All root tips were examined using a stereomicroscope (Leica M205 FA, Wetzlar, Germany) and assigned to one of the following root fractions: vital ectomycorrhizal (EM), vital non-ectomycorrhizal (NM), dead ectomycorrhizal (EM_{dry}) and dead non-ectomycorrhizal (NM_{dry}). Live and dead EM or NM root tips were sorted as described by Winkler et al. (2010), Downes (1992). EM root tips were described and categorized morphologically, by color, mantle surface, branching, presence of hyphae and rhizomorphs, following the procedure and identification keys of Agerer (1987-1996). The occurring morphotypes were numbered and photographed (Leica DFC 420 C, Wetzlar, Germany). For each morphotype, 10 to 20 root tips were collected and kept frozen at -80°C until fungal molecular identification

3.2.5. Collection of root tips for isotope analysis

Amongst the ten morphotypes found, five were frequent enough in each treatment to be collected for isotope analysis. Depending on EM morphology, 20 to 60 root tips were required for a suitable sample for isotope analysis. One replicate comprised the root tips obtained from one individual plant. EM morphotypes and other classes of root tips of each plant were cut under the compound microscope. Two different sets of instruments were used to handle with non-labeled and labeled plants to avoid cross contamination. EM tips were excised at the level of last lateral root ramification, ensheathed by the mantel; NM tips were sampled at the level of the youngest and active "white" zone (Enstone et al 2001; Evert and Eichhorn 2007); dead root tips were cut at the same position as NM tips. Samples were freeze dried for isotope analysis.

3.2.6. Molecular identification of EM fungal species

Frozen root tips samples were ground in liquid nitrogen using a ball mill (type MM2, Retch, Hann, Germany) to facilitate DNA extraction. Total genomic DNA isolation was achieved using a plantDNA-OLS kit (OLS OMNI Life Science, Hamburg, Germany) according to the manufacturer's instructions. Isolated DNA was used as a template for PCR amplification of internal transcribed

spacer (ITS) region of the fungal ribosomal DNA. We used the primer pairs ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3'), or ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4, after White et al. (1990). The PCR reaction mixture consisted of the following components: 2.5 µl 10X Taq buffer with $(NH_4)_2SO_4$ (Fermentas, St-Leon-Rot, Germany); 3.0 µl 25 mM $MgCl_2$; 0.5 µl 10 mM dNTP mix (Fermentas, St-Leon-Rot, Germany); 1.25 µl 10 mM ITS5; 1.25 µl 10 mM ITS4 (MWG, Biotech, Ebersberg, Germany); 0.125 µl (5u μl^{-1}) Taq DNA Polymerase (Fermentas, St-Leon-Rot, Germany); 14.375 µl nuclease-free water (Ambion Inc.,Austin, USA); 2 µl template DNA. PCR experiments were performed in a Mastercycler gradient thermal cycler (Ependorf, Hamburg, Germany). The reaction was started by 1 min denaturation at 95°C, afterwards 35 cycles were run: denaturation at 95°C for 30 s, anneling at 55°C for 30 s and extention to 72°C for 1 min; the reaction was finished by a final extension step at 72°C for 5 minutes and followed a decrease in temperature to 4°C. PCR products were separated by electrophoresis, on a 1.5% agarose gel supplemented with ethidium bromide and were visualised under UV light (Fluor-STM Multiimager, BioRad, Munich, Germany) as described by Sambrook J. et al. (2001). Single band PCR products were submitted to further DNA sequencing. The PCR products were purified using straightPCR-OLS kit (OLS OMNI life science, Hamburg, Germany), following the instructions of the manufacturer. When PCR resulted in more than one amplification product, the fragment with the expected size was cloned into a pGEM-T vector (pGEM-T System I Promega, Madison, USA). Selection has been performed by a blue-white assay on LB agar plates, supplemented with ampicillin (100 µg ml^{-1}). The transformants were analyzed for the presence of plasmid DNA, by ITS region re-amplification, as described above. PCR products of expected size were purified by precipitation with isopropanol for 1 hr at room temperature, followed by 30 minutes centrifugation, 17900 g, at room temperature (centrifuge 5417 R Eppendorf, Hamburg, Germany) and sequenced. Sequencing was performed by the dideoxy chain – termination method, described by Sanger et al. (1977), using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Foster City, USA). Further, the sequencing was carried out in the Department of Forest Genetics, Buesgen-Institute, University of Goettingen, using and an automatic sequencer (ABI Prism 3100 gEnetic Analyser, 36 cm capillary, Matrix Pop 6, Applied Biosystems Foster City, USA) under conditions recommended by manufacturer.

Sequences were assembled using Staden Package 4.10 (http://staden.sourceforge.net). For fungal identification, BLAST searches were carried out against the NCBI (http://www.ncbi.nlm.nih.gov/) and UNITE (http://unite.ut.ee) public sequence databases. Sequences were assigned with matching species names when the BLAST matches found have showed identities higher than 98%. The

sequences have been deposited in NCBI GenBank with the GenBank accession numbers HM748636 – HM748643.

3.2.7. EM anatomy

EM species, NM, DM and DR root tips were collected from control trees and fixed in 7% formaldehyde, 18% ethanol and 19.2% of acetic acid. Samples were dehydrated and embedded in styrene-methacrylate after a protocol described by Ducic et al. (2008). One-µm thick cross sections were cut with a sliding microtome (Reichert-Jung, Vienna, Austria). The sections were mounted on gelatinized microscopic slides and stained with 0.1% toluidine blue in 0.1% di-sodium tetra borate for 3 to 5 min. Sections were photographed under a microscope (Axioplan, Carl Zeiss AG, Germany) using a digital camera (AxioCam MRc, Carl Zeiss AG, Germany).
Fungal mantel volume (V) was calculated as follows:

$$V_{mantel} = \pi\, h\, R^2_{root\ tip} - \pi\, h\, (R_{root\ tip} - R_{cortex\ and\ stele})^2$$

Where, h is the mean length and R the radius of EM colonized root tips, measured using the stereomicroscopic photos; $R_{cortex\ and\ stele}$ was obtained by measured the cross sections.

3.2.8. Isotope analysis

Freeze-dried root tips were weighed using a super-micro balance (S4, Sartorius, Göttingen, Germany). A suitable sample for isotope estimation consisted in 0.2 to 1.1 mg dry mass, what represented about 20 to 60 root tips. Total C, N and ^{15}N concentrations in root tips were determined at the Centre for Stable Isotope Research and Analysis (University Göttingen) with an isotope ratio mass spectrometer (IRMS Delta ^{plus}, Thermo Finnigan Mat, Bremen, Germany) coupled to an elemental analyzer (EA 1108, Fisons, Rodano, Italy).

3.2.9. Calculations and statistical analyses

3.2.9.1 ^{15}N measurements

The relative ^{15}N abundance was expressed as the ratio:

$$^{15}N\ (\text{atom }\%) = \frac{^{15}N}{^{14}N + ^{15}N} \times 100$$

^{15}N content per root tip was calculated as follows:

$$^{15}N \text{ content (ng)} = [\text{biomass (g)} \times \text{N concentration (ng g}^{-1}) \times {}^{15}\text{N atom \% excess}] / 100$$

where the biomass represents the mean dry mass of one root tip, according with colonizing EM fungal species, and

$$^{15}N \text{ Atom \% excess} = \text{Atom \%}_{\text{labeled}} - \text{Atom \%}_{\text{unlabelled}}$$

where labeled and unlabelled refers to samples affected by application of ^{15}N label and samples that contain only natural concentrations of N isotopes, respectively.

3.2.9.2 Statistical analysis

Statistical analysis was performed using Statgraphics Plus 3.0 (StatPoint, Inc., St Louis, MO, USA). When necessary, data were transferred logarithmically or by square root to satisfy the criteria of normal distribution and homogeneity of variance. When transformation of the data did not meet these requirements, the Kruskal-Wallis and Mann-Whitney W tests were applied instead of analysis of variance (ANOVA). Means or medians were considered to be significantly different from each other, if $P \leq 0.05$.

3.3. Results

3.3.1. Root demography and EM community structure are influenced by irradiance and water availability

EM colonized and non-colonized beech seedlings showed a significant difference in the number of root tips (P = 0.001, F = 28.98). The presence of EM fungi stimulated root tip formation (Table 3.1). Full irradiance also increased the number of root tips, but less than the presence of EM fungi (P = 0.015, F = 6.19). Reduced water availability increased the number of root tips (P = 0.024, F = 5.31). We observed a higher increment of the number of root tips for plants grown under drought in combination with full light than for those grown under drought and shade (Table 3.1).

The pattern of distribution of different root fractions was maintained under all treatments: EM ranged from 30 ± 7 to 39 ± 3.3 %. NM root tips were the major fraction, ranging from 39 ± 2 to 51 ± 5%,

DR 15 ± 2 to 20 ± 3%, and DM was the smallest fraction of about 4 ± 1% (Table 3.1). The fraction of NM root tips decreased in response to reduced water availability for EM plants (P = 0.035, F = 4.64), whereas the fraction of EM colonized root tips increased slightly (Table 3.1).

To characterize EM fungal community associated with roots of EM plants, we conducted morphotyping and ITS sequencing. We identified ten fungal taxa: *Cenococcum geophilum*, *Tuber rufum*, *Tuber* sp2, *Tomentella punicea*, *Tuber* sp1, *Tomentella badia*, UEM Telephoraceae, UEM Sebacina, UEM Cortinarius, *Tomentella viridula* (Tabel 3.2). A description of the morphotypes, the results of ITS sequencing and the GenBank accession number are given in Table 3.2. A photo of each fungal taxon is included in Supplement 3.1.

Table 3.1: Root demography: number of root tips and the percentages represented by different root fractions: non-myccorhizal (NM), ectomycorrhizal (EM), dry non-mycorrhizal (Dry-NM) and dry ectomycorrhizal (Dry-EM) root tips; under two levels of irradiance and water availability (LC, full light control; LD, full light drought; SC, shade control; SD, shade drought). Mean values ± SE (n = 10, n = 5 for NM plants in LC and LD treatments). Below: statistical effects of ectomycorrhizas presence (EM), irradiance (L) and water availability (D) on total number of root tips and percentages of different root fractions assessed by ANOVA multi-factors analysis. P ≤ 0.05 are shown by bold letters.

Growth regimes	Number of root tips	NM root tips (%)	EM root tips (%)	Dry-NM root tips (%)	Dry-EM root tips (%)
EM-LC	5365 ± 675	51 ± 4	30 ± 7	15 ± 1	4 ± 1
EM-LD	7315 ± 754	48 ± 3	32 ± 11	15 ± 1	4 ± 1
EM-SC	4943 ± 658	45 ± 2	31 ± 1	20 ± 2	3 ± 1
EM-SD	5530 ± 722	39 ± 1	39 ± 3	18 ± 3	4 ± 1
NM-LC	2831 ± 321	89 ± 3	0 ± 0	11 ± 3	0 ± 0
NM-LD	4644 ± 717	75 ± 5	0 ± 0	23 ± 5	0 ± 0
NM-SC	2524 ± 275	87 ± 2	0 ± 0	13 ± 2	0 ± 0
NM-SD	2723 ± 251	85 ± 4	0 ± 0	15 ± 4	0 ± 0

	F	P	F	P	F	P	F	P	F	P
EM	28.98	**0.001**	-	-	-	-	-	-	-	-
L	6.19	**0.015**	0.40	0.527	1.25	0.270	0.03	0.865	0.00	0.985
D	5.31	**0.024**	4.64	**0.035**	1.61	0.212	0.97	0.329	0.88	0.354

Table 3.2: Morphological and anatomical characterization of EM morphotypes formed with roots of young beech plants. For morphotypes identified by ITS sequencing, the GenBank accession number, data of best alignment by BLAST in UNITE (accession numbers starting with UDB) or NCBI (other accession numbers) are indicated. Obtained nucleotide sequences have been deposited in the NCBI GenBank database under accession numbers HM 748636- HM748642.

Species	Best BLAST match(es) (% similarity)	Description
Cenococcum geophilum		Single or rarely dichotomously branched, black, compact mantel, black rigid hyphae emanating from mantel. Mantel width: 30.0µm (compact cells)
Tuber rufum HM748641	UDB000996: *Tuber rufum* (99%) FN433168: *Tuber rufum* (99%)	Ramification monopodial-pinnate, light brown, rare whitish emanating hyphae Mantel width: 22.34µm (loose cells)
Tuber sp 2 HM748637	UDB001385: *Tuber puberulum* (92%) FM205642: *Tuber puberulum* (99%)	Single or monopodial branched, brown orange, abundant short with similar lengths emanating hyphae Mantel width: 20.5µm
Tomentella punicea HM748638	UDB000950: *Tomentella punicea* (99%) AY299299: Unculture Tomentella (99%)	Monopodial irregularly branched, brown, few emanating hyphae Mantel width: 36.8µm

Species	Best BLAST match(es) (% similarity)	Description
Tuber sp 1 HM748636	UDB000121: *Tuber maculatum* (89%) EU668241: Uncultured Tuber isolate (99%)	Monopodial-pinnate, light brown, with short irregular hyphae Mantel width: 28.8μm
UEM Sebacina HM748642	UDB000975 *Sebacina epigaea* (96%) EU668267 Uncultured *Sebacina* (98%)	Monopodial ramified, brown yellowish, with white long emanating hyphae
Cortinarius like	Sequencing not successful	Irregularly pinnate ramified, brown-reddish, with abundant white emanating hyphae. Rhizomorphs are present
Tomentella badia HM748639	UDB000952: *Tomentella badia* (98%) GQ478014: Uncultured Tomentella (96%)	Monopodial, brown, with only few emanating hyphae
UEM Telephoraceae HM748640	UDB003313: *Tomentella* (94%) DQ150126:	Monopodial-pinnate or monopodial-pyramidal, black, with rigid, relative abundant, yellow brownish hyphae.

Species	Best BLAST match(es) (% similarity)	Description
	Uncultured Thelephoraceae (95%)	
Tomentella viridula HM748643	UDB000261: *Tomentella viridula* (99%) EU816647: Uncultured Thelephoraceae (100%)	Monopodial- dichotomously branched, dark green-brownish with lighter ends, with short white emanating hyphae

Relative abundance of different EM fungal species varied between treatments (Tabel 3.3). *Cenococcum geophilum* had a significantly lower abundance in association with roots of plants grown under drought, compared with those of well irrigated plants. Overall, the abundance of *C. geophilum* ranged considerably from 2.8 to 83.3% (Table 3.3). The abundance of *Tuber rufum* was more than two fold higher in full light than in shade, but as it did not occur on the roots of all investigated plants, no significant differences between the treatments were found (Table 3.3). *Tuber* sp2 showed a higher frequency in associations with roots of plants grown in shade than of those grown in full light (Table 3.3). *Tomentella punicea* revealed similar frequencies under all four treatments, only with a slight increment under drought in shade. The relative frequency of *T. punicea* was generally the greatest of all fungi (Table 3.3). *Tuber* sp1 displayed similar frequencies between treatments (Table 3.3). The other five fungal species, UEM Sebacina, UEM Cortinariaceae, *Tomentella badia*, UEM Telephoracae, and *Tomentella viridula*, displayed very low frequencies, with only 1.5 % of the EM root tips colonized per fungal species. Together they colonized less than 5% of the EM root tips per treatment. However, all ten species occurred in each treatment.

Table 3.3: Relative abundances of ectomycorrhizal fungal species associated with the roots of ectomycorrhizal-colonized plants grown under full light-control (LC); full light-drought (LD); shade-control (SC); shade-drought (SD). Mean values; in brackets: minimum and maximum value (n=10). Different letters in columns indicate significant differences at $P \leq 0.05$, obtained by Mann-Whitney (Wilcoxon) W tests to compare medians.

Fungal taxon	LC	LD	SC	SD
Cenococcum geophilum	23.3^{b} (9.4; 44.1)	12.2^{a} (2.8; 25.7)	25.4^{b} (7.3; 83.3)	19.1^{ab} (2.4; 75.5)
Tuber rufum	25.0^{a} (0.0; 74,2)	15.9^{a} (0.0; 75.0)	5.9^{a} (0.0; 29.5)	6.3^{a} (0.0; 17.4)
Tuber sp 2	6.5^{a} (0.0; 29.6)	6.6^{ab} (0.5; 75.0)	12.0^{b} (0.9; 19.2)	9.2^{b} (3.0; 21.0)
Tomentella punicea	21.8^{a} (0.0; 58.0)	32.9^{ab} (6.2; 70.1)	33.3^{ab} (0.0; 58.1)	45.3^{b} (0.0; 78.8)
Tuber sp 1	18.3^{a} (5.9; 39.8)	25.6^{a} (0.0; 50.6)	18.4^{a} (0.0; 53.7)	17.2^{a} (2.5; 32.9)
Tomentella badia	0.2^{a} (0.0; 0.9)	4.6^{b} (0.0; 20.9)	1.5^{b} (0.0; 4.8)	0.6^{ab} (0.0; 1.8)
UEM *Telephoraceae*	0.7^{a} (0.0; 3.0)	1.3^{a} (0.0; 4.6)	1.4^{a} (0.0; 3.6)	1.2^{a} (0.0; 7.7)
UEM *Sebacina*	1.1^{a} (0.0; 5.4)	0.5^{a} (0.0; 2.9)	0.3^{a} (0.0; 1.1)	0.3^{a} (0.0; 1.8)
UEM *Cortinariaceae*	1.9^{b} (0.0; 9.0)	0.3^{ab} (0.0; 1.5)	0.1^{a} (0.0; 0.8)	0.2^{a} (0.0; 2.3)
Tomentella viridula	1.0^{a} (0.0; 6.2)	0.2^{a} (0.0; 1.3)	1.8^{a} (0.0; 4.0)	0.7^{a} (0.0; 4.4)

Shannon-Wiener index (H′), as well as Evenness were not influenced by water availability or irradiance (Tabel 3.4).

The rare fungal species, UEM *Sebacina*, UEM *Cortinariaceae*, *Tomentella badia*, UEM *Telephoraceae*, and *Tomentella viridula*, were called other EMs and were not included in C and N analysis because of their sparseness.

Tabel 3.4: Shannon-Wiener index (H') and Evenness of ectomycorrhizal fungal species associated with the roots of ectomycorrhizal colonized plants grown under two levels of irradiance and water availability (LC, full light control; LD, full light drought; SC, shade control; SD, shade drought). Mean values ± SE (n = 10). Statistical effects of, irradiance (L) and water availability (D) on Shannon-Wiener index (H') and Evenness assessed by ANOVA multi-factor analysis. P ≤ 0.05 are shown by bold letters.

	LC	LD	SC	SD	L		D	
					F	P	F	P
H'	1.37 ± 0.08	1.29 ± 0.07	1.34 ± 0.12	1.24 ± 0.12	0.00	0.968	0.49	0.488
Evenness	1.87 ± 0.08	1.96 ± 0.08	1.89 ± 0.15	1.94 ± 0.08	0.34	0.564	2.63	0.113

3.3.2. Ecomycorrhizal fungal species influence ^{15}N contents in root tips

To find out if functional differences existed in N accumulation amongst EM and NM root tips, we determined the ^{15}N content root tips according to fungal species and root colonization. The ^{15}N content of individual EM fungi were investigated under two levels of irradiance and water availability (Figure 3.1A, B, C, D). The root tips of the well watered plants grown in full light had the highest content of ^{15}N in association with *T. rufum*, followed by those associated with *Tuber* sp 1 (Figure 3.1A). Under this growth regime, the NM root tips and those colonized by *C. geophilum* and *T. punicea* had significantly lower ^{15}N contents than root tips colonized by *T. rufum* and *Tuber* sp 1 (Figure 3.1A). Dead root tips, both EM and NM ones, showed the lowest ^{15}N content, close to zero (Figure 3.1A).

Drought stress applied to the plants grown in full light induced several changes in the pattern of ^{15}N contents in the root tips (Figure 3.1B). NM root tips continued to have significantly smaller ^{15}N contents than those of other categories of root tips. The root tips colonized by *T. rufum* still contained the highest ^{15}N amounts, while the root tips colonized by *T. punicea* and those by *C. geophilum* revealed significantly greater contents than NM root tips. ^{15}N contents of root tips colonized by *Tuber* sp1 were not anymore higher than those of other root tips. This was also true in comparison with those of well watered plants grown in the full light (Figure 3.1B).

The root tips of well watered plants grown in shade contained lower amounts of ^{15}N compared with those of plants grown under full light (Figure 3.1A, C). Under these conditions, the ^{15}N contents of root tips colonized by *Tuber* sp1, *T. punicea*, *Tuber* sp2 were in the range of those of dead root tips, indicating that ^{15}N uptake was close to zero. Only NM root tips and those colonized by *T. rufum* and *C. geophilum* revealed still significant effectiveness for ^{15}N uptake (Figure 3.1C).

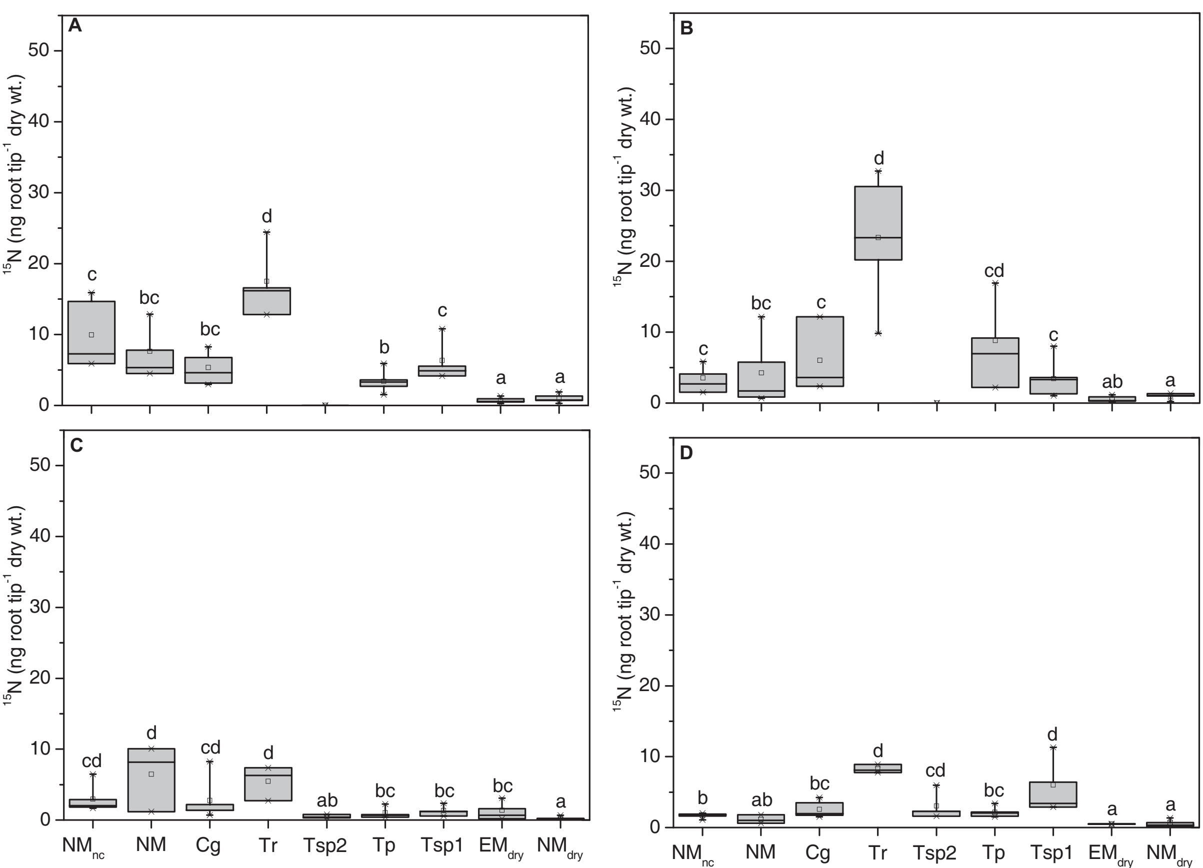

Figure 3.1: ^{15}N accumulated in non-mycorrhizal and ectomycorrhizal root tips of beech seedlings grown under well irrigated conditions in light (A) and in shade (C) and under drought in light (B), and in shade (D). Ectomycorrhizal tips were separated by fungal species: *Cenococcum geophilum* (Cg), *Tuber rufum* (Tr), *Tuber* sp 2 (Tsp2), *Tomentella punicea* (Tp), *Tuber* sp 1 (Tsp1). Other fractions are: non-mycorrhizal root tips of non-colonized plants (NM_{nc}), non-mycorrhizal root tips of colonized plants (NM), dry ectomycorrhizal (EM_{dry}) and dry non-mycorrhizal (NM_{dry}). n=5; in very few cases n=4; in low irradiance-control for Tr, Tsp2 and NM n=3; in low irradiance-drought for Tr n=3. Different letters indicate significant differences at $P \leq 0.05$ between root tips categories in each treatment.

Increments in ^{15}N in the EM root tips were stimulated by reduced water availability in shade-grown plants (Figure 3.1 D), whereas ^{15}N contents in NM root tip decreased close to zero. Under these conditions, the greatest amounts of ^{15}N were found in the root tips colonized by the three species of Tuber, *T. rufum*, *Tuber* sp 1 and *Tuber* sp 2 (Figure 3.1 D). In each treatment, *Tuber rufum*-colonized root tips that had the highest ^{15}N content compared with those associated with other fungal species or NM root tips (Figure 3.1A, B, C, D). ^{15}N contents in the root tips of non-colonized plants were not significantly different from Nm root tips of EM plants (Figure 3.1A, B, C, D).

The NM root tips of non-colonized plants showed ^{15}N contents similar to those of NM root tips of EM colonized plants, in each treatment.

3.3.3. Total N contents in ectomycorrhizal and non-mycorrhizal root tips show a different pattern according to fungal species as compared to ^{15}N contents

We measured the total N amounts in the root tips colonized by different EM fungi and in NM root tips to find out whether newly absorbed ^{15}N is representative for overall N accumulation in root tips. The root tips of well watered plants grown in full light colonized by *Tuber* sp 1 had the greatest total N contents compared with those colonized by other fungi or NM root tips (Figure 3.2A). Under the same conditions, root tips associated with *T. rufum* and *T. punicea* displayed also significantly larger contents of total N, than those colonized by *C. geophilum* and NM root tips (Figure 3.2A).

Drought stressed plants in full light contained significantly lower amounts of total N in NM root tips than in EM root tips. Despite of the lowest N amounts in the root tips colonized by *C. geophilum* in well watered plans, these root tips contained increased amounts of N under drought stress (Figure 3.2B). However, shade decreased this effect. In both, well watered and drought stressed plants, the root tips colonized by *C. geophilum* had the lowest total N contents (Figure 3.2 C, D). In shade, NM root tips showed intermediate N contents between those of *C. geophilum* and other EM root tips (Figure 3.2 C, D). Root tips colonized by *T. rufum* of well watered plants had significantly largely amounts of N than NM root tips or those colonized by other fungi, except the root tips associated with *T. punicea* (Figure 3.2C). After application of drought stress, root tips colonized by *T. rufum*, *T. punicea* and *Tuber* sp 1 displayed higher total N contents than those colonized by *C. geophilum* and *T. punicea*; NM root tips had significantly greater amounts of N than the latter ones, but smaller than the root tips colonized by *T. rufum*, *T. punicea* and *Tuber* sp 1 (Figure 3.2 C).

In each treatment, N contents of root tips varied between different EM, but were similar between NM root tips of non-colonized plants and those of EM colonized plants.

3.3.4. ^{15}N to N ratio vary with ecomycorrhizal fugal species and with the growth regime

Functional differences amongst EM fungal species within the community were investigated by measuring ^{15}N to N ratio in root tips colonized by different EM fungi, as well as in NM root tips.

Tuber rufum was the fungus with the highest positive impute on ^{15}N to N ratio in root tips, in each treatment, but especially in full light conditions (Figure 3.3 A, B, C, D). ^{15}N to N ratio varied in other fungi with the growth regime. For example, *T. punicea* displayed a lower ratio in root tips of well watered plants grown in full light, compared with those colonized by other fungi or NM root tips

(Figure 3A), while in drought stressed plants grown in light, *T. punicea*-colonized root tips showed an increased ^{15}N to N ratio compared with other root tips (Figure 3.3B). In shade, *T. punicea* did not increase the ^{15}N to N ratio, compared with other fungi (Figure 3.3C, D)

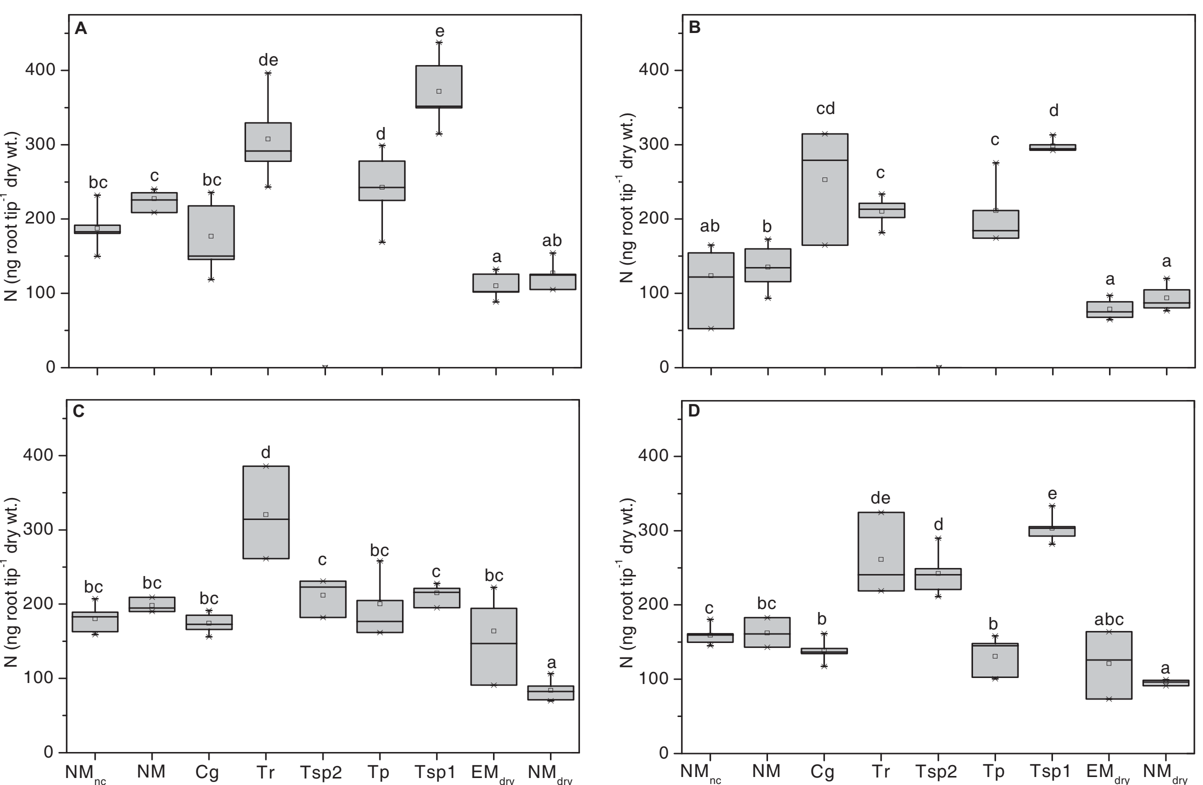

Figure 3.2: N amount in non-mycorrhizal and ectomycorrhizal root tips of beech seedlings grown under well irrigated conditions in light (A) and in shade (C) and under drought in light (B), and in shade (D). Ectomycorrhizal tips were separated by fungal species: *Cenococcum geophilum* (Cg), *Tuber rufum* (Tr), *Tuber* sp 2 (Tsp2), *Tomentella punicea* (Tp), *Tuber* sp 1 (Tsp1). Other fractions are: non-mycorrhizal root tips of non-colonized plants (NM_{nc}), non-mycorrhizal root tips of colonized plants (NM), dry ectomycorrhizal (EM_{dry}) and dry non-mycorrhizal (NM_{dry}). n=5; in very few cases n=4; in low irradiance-control for Tr, Tsp2 and NM n=3; in low irradiance-drought for Tr n=3. Different letters indicate significant differences at $P \leq 0.05$ between root tips categories in each treatment.

Root tips of drought stressed plants grown in shade colonized with *Tuber* sp1 showed higher ^{15}N to N ratio than those of drought stressed plants in full light, or the well watered plants grown in shade. The effects of *Tuber* sp 2 were measured only in the root tips of plants grown in shade, because the fungus was missing in the other treatments. We observed that the root tips of drought stressed plants colonized by *Tuber* sp2 had an approximately 10 fold higher ^{15}N to N ratio than those of well watered plants (Figure 3.3C, D). In each treatment, the ^{15}N to N ratio in root tips colonized by *C.*

geophilum was similar to that of NM root tips. NM root tips showed moderate N turnover in full light, while in shade strong differences between well watered and drought stressed plants appeared.

In well watered plants grown in light, in root tips colonized by *Tuber* sp1, *T. punicea* and *Tuber* sp2 the ^{15}N to N ratios were in the range of those of dead root tips (Figure 3.3C).

NM root tips displayed the highest N turnover in well watered plants and the lowest, close to zero, in drought stressed plants compared with other root tips categories (Figure 3.3A, B, C, D). There were no significant differences in ^{15}N to N ratio, in each growth regime, between NM root tips of non-colonized plants and those of EM colonized plants.

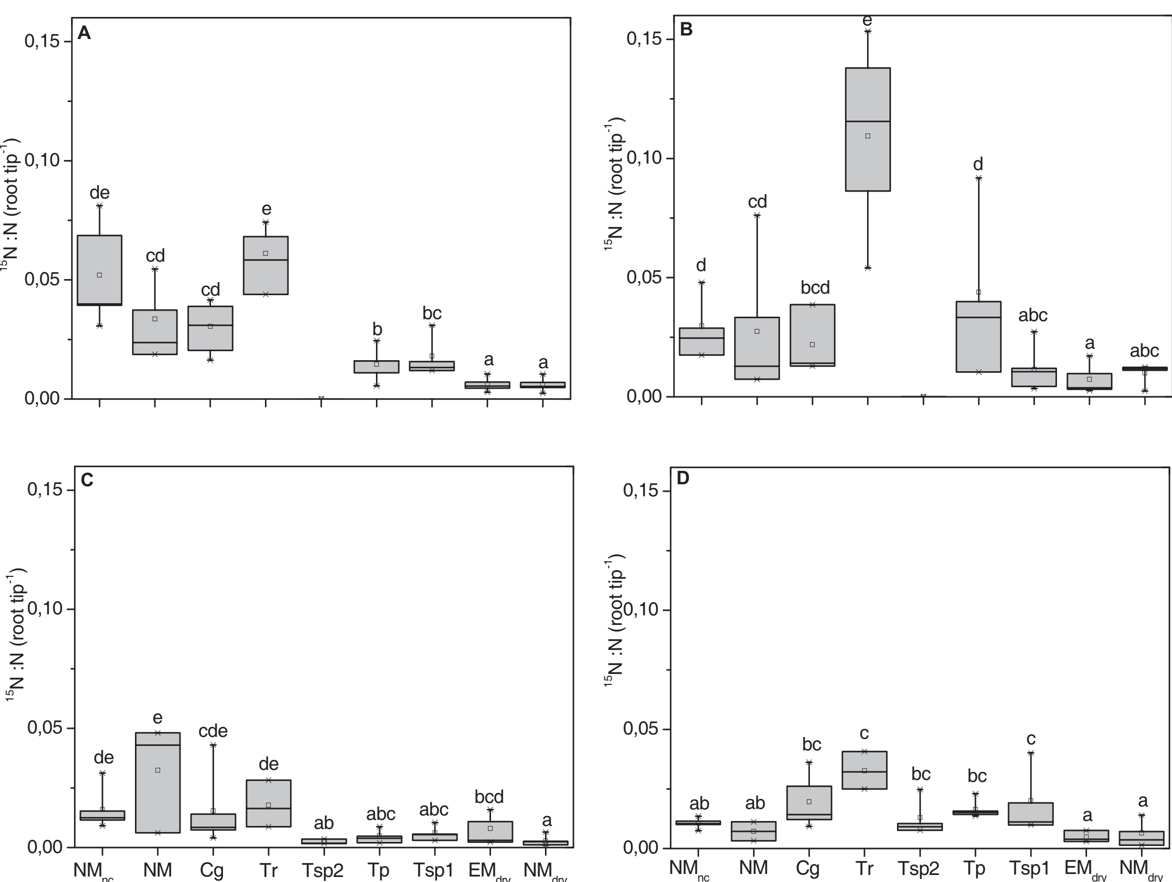

Figure 3.3: ^{15}N to N ratio of non-mycorrhizal and ectomycorrhizal root tips of beech seedlings grown under well irrigated conditions in light (A) and in shade (C) and under drought in light (B), and in shade (D). Ectomycorrhizal tips were separated by fungal species: *Cenococcum geophilum* (Cg), *Tuber rufum* (Tr), *Tuber* sp 2 (Tsp2), *Tomentella punicea* (Tp), *Tuber* sp 1 (Tsp1). Other fractions are: non-mycorrhizal root tips of non-colonized plants (NM_{nc}), non-mycorrhizal root tips of colonized plants (NM), dry ectomycorrhizal (EM_{dry}) and dry non-mycorrhizal (NM_{dry}). n=5; in very few cases n=4; in low irradiance-control for Tr, Tsp2 and NM n=3; in low irradiance-drought for Tr n=3. Different letters indicate significant differences at $P \leq 0.05$ between root tips categories in each treatment.

3.3.5. ^{15}N sequestrations in root tips is negatively correlated with fungal mantle

To find out if N metabolism of EM root tips was related to the fungal biomass, ^{15}N, N amounts and ^{15}N to N ratio of the root tip were correlated with fungal mantle volume. Regression analysis showed a negative relation between fungal mantle volume and ^{15}N content per EM root tip (R = -0.38, P < 0.001), and ^{15}N to N ratio of the root tip (R = -0.42, P < 0.001), and no correlations with N content per EM root tip (R = 0.02, P = 0.846, Figure 3.4).

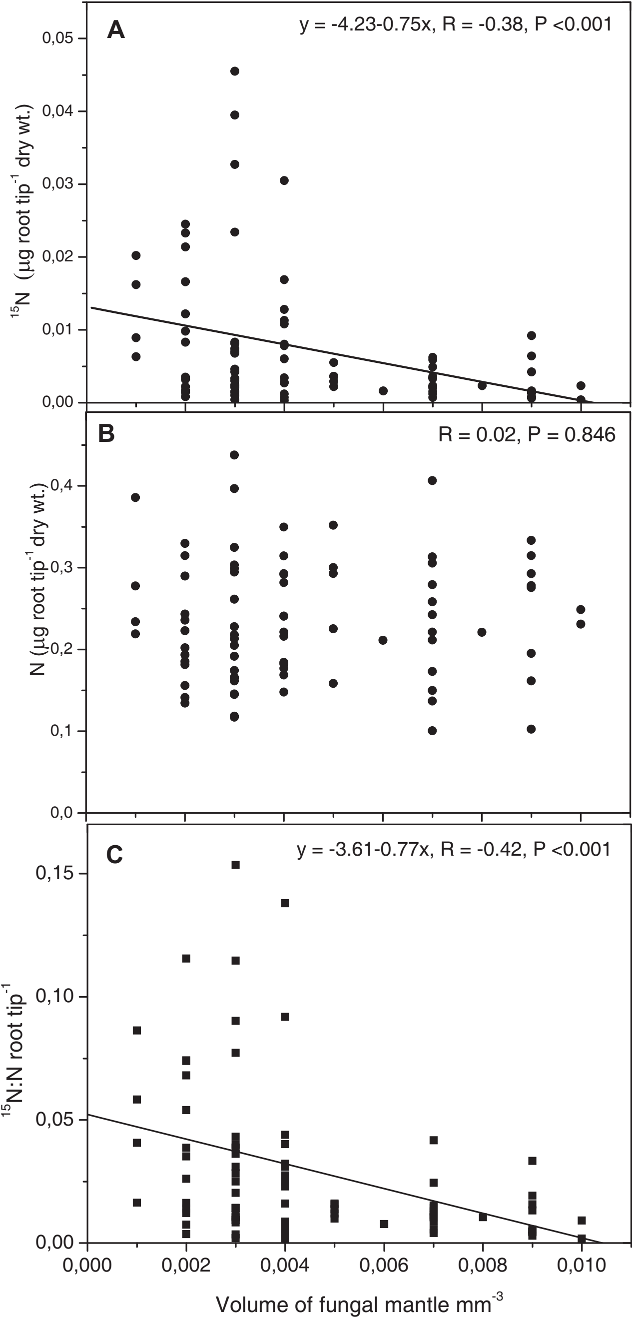

Figure 3.4: Regression analysis between the volume of fungal mantle and the amount of ^{15}N per ectomycorrhizal root tip (A), the amount of N per ectomycorrhizal root tip (B) and ratio ^{15}N:N of ectomycorrhizal root tip (C).

3.4. Discussion

3.4.1. EM community structure under shade and drought

The EM fungal community found on young beech plants of this study comprised ten species with relative frequencies depending on growth regimes. The number of species described in the investigated EM community is similar to that observed in natural EM communities associated with young plants. For example, Kranabetter and Wylie (2001) found that roots of 4-years old seedlings grown in gap forest were colonized by up to 11 EM fungal species. Dahlberg (2001) suggested that plants that can access only small soil volumes such as seedlings contain usually only few species of EM. All EM fungal species found here were described on beech (Buee et al. 2005, Pena et al. 2010). We focused on five EM fungal species that colonized about 95% of root tips, *Cenococcum geophilum*, three *Tuber* species and *Tomentella punicea*. Regarding their external mycelia and soil exploration strategies, *C. geophilum* and *Tuber* species are classified as short distance type and *T. punicea* as medium-distance smooth type (Agerer 2001). *T. punicea* was the only fungal species in the investigated community that has the capacity to form rhizomorphs (Agerer 2001). Fungal species possessing long hyphae and rhizomorphs are suitable for absorbing and transferring water and solutes over relatively long distances (Duddridge et al. 1980, Plamboeck et al. 2007). In our experiment, *T. punicea* displayed the highest frequency in association with the roots of drought stressed plants in shade compared with plants exposed to other treatments.

EM community structures are influenced by drought stress (Gehring et al 1998, Nilsen et al. 1998, Shi et al. 2002). Maintenance of fungal species that have the capacity to develop rhizomorphs was also reported by Shi et al. (2002). *C. geophilum* is a drought-resistant species than can resist at very low water potentials (Coleman et al. 1989, Buee et al. 2005, di Pietro et al. 2007), but in our study, its frequency decreased significantly under drought stress. Probably, the competitiveness of *C. geophilum* is higher at lower levels of soil moisture rather than at intermediate levels where other EM fungi are still competitive active (Kennedy et al 2007). Thus, its abundance may increase under severe drought. *T. rufum* colonized a smaller percentage of root tips in shade compared with full light. Similar results were reported by Druebert et al. (2009), who found that *T. rufum* colonized up to 17.2 % of root tips of sun beech, but did not appear on roots of shade beech. C limitation suppresses morphotypes with thick mantels in the advantage of those with thin mantels (Markkola et al. 2004). However, *T. rufum* had a lower mantel thickness than *C. geophilum* or *T .punicea*, which appeared with a higher frequency in shade. Therefore, the reduced frequency of *T. rufum* in shade may not only be caused by reduced C delivery for its own growth. These results indicate that the

structure of the EM community associated with young beech plants depends on environmental variables such as light and water availability. We showed that fungi as *T. punicea* and *C. geophilum* are favored by shade and *T. punicea* by drought.

3.4.2. N turnover in EM and NM root tips

Our results show differences amongst EM fungi for N turnover in the colonized root tips. By a non-invasive experiment, the behavior of different EM fungi with respect to N turnover in the colonized root tips, within community, was investigated in relation with light and water availability. Both these environmental factors can directly affect the EM fungal competitiveness by reduction of C supply.

Root tips associated with *T. rufum* had the highest ^{15}N content in each treatment. However, similarly with its higher frequency in full light than in shade, *T. rufum* was functionally more active in light than in shade. As *T. rufum* has a thin mantle and no capacity to form extensive eternal mycelia, with long hyphae or rhizomorphs, the C requirements to build its own biomass has to be low. Still it showed preference for full light conditions. This may be explained by a higher C need necessary for its intense N processing. Compared with other fungi of EM community, *T. rufum* induced also higher ^{15}N contents in root tips of plants grown in shade. The lower frequency of *T. rufum* in shade than in full light might point to a disorder of C - N coupling for N export to the plant. Over accumulation of N may generate toxicity. Due to the small mantle volume, *T. rufum* has probably a low capacity to sequester N in vacuolar bodies within the mantle to avoid toxicity (Kottke et al. 1995).

N turnover in EM root tips of well watered plants grown in shade was lower than in those of plants grown in full light. On the contrary, NM root tips showed a higher N turnover in shade than in full light. These differences between N turnover in EM and NM root tips may be caused by differences in C need. N metabolism in EM root tips might have been more C-limited than in NM ones, due to the mandatory energy supply to the fungi. Furthermore, the differences in N turnover found in different fungal species may be the result of different responsiveness to changes in carbohydrate supply of EM fungal species (Kuikka et al. 2003, Markkola et al. 2004, Saravesi et al. 2008, Druebert et al. 2009, Pena et al. 2010).

N turnover in the root tips of drought stressed plants in shade increased in EM root tips compared with NM ones. This suggests that EM hyphae have the capabilities to forage for N, which becomes immobile and inaccessible under lower levels of soil moisture (Azcon et al. 1996, Azcon et al. 2008). The greatest difference between ^{15}N sequestration in root tips in well watered and drought stressed plants were observed in the root tips colonized by *C. geophilum*, *T. punicea* or *Tuber* sp 2. Nonetheless, in contrast with the latter two EM fungi, *C. geophilum* did not reveal a higher N

turnover under drought than under well watered conditions. *C. geophilum* contained large amounts of total N, likely deposited in vacuolar bodies (Kottke et al. 1995).

Despite the fact that N turnover in root tips colonized by different EM fungi varied between treatments reflecting different physiological abilities of the colonizing fungus, we observed overall a common pattern of fungal behavior with respect to growth regime. N turnover in beech root tips was higher in full light than in shade and under drought stress than under well watered conditions. In full light, both in well watered and drought stressed plants, NM root tips revealed intermediate values of N turnover compared with those of EM root tips. While in shade, the N turnover in NM and EM root tips differ strongly with water availability. In well watered plants in shade, the majority of EM root tips showed no N turnover. While, in drought stressed plants in shade, N turnover in NM root tips was close to zero and much increased in EM root tips. Shade and drought, both limiting C-flux to the roots, and their combination shade-drought caused a shift between NM and EM root tips, for N turnover. In shade, EM fungal species appeared to be less physiologically active for N uptake and turnover, but in shade combined with drought, they showed the contrary. These results can be explained either by counteracts of the C limitation to EM root tips to couple the N, and this has to be under host plant control, or by alteration under drought of rates of nutrient supply and partitioning of resources between soil microorganisms and plant roots (Winkler et al. 2010).

Hobbie and Agerer (2010) suggested that the amounts of N sequestrated as immobile N in fungal biomass are negatively correlated with the quantity of metabolically active N that has to be transported to the plant. We have found a negative correlation between fungal mantle volumes and amounts of ^{15}N in the root tip. This resulted also in a strong negative correlation with N turnover. Despite of a large nutrient absorbance surface, the EM fungal species of high biomass, with extensive external mycelia appeared to have a lower N turnover than low biomass EM fungi.

3.5. Conclusions

This study lends supports to the concept of functional diversity of EM fungal species with respect to N uptake and processing. They were clear differences in N turnover in the root tips colonized by different EM fungal species. Moreover, EM fungi such as *T. punicea*, the only fungus that, theoretically, can form rhizomorphs, showed higher effectiveness for ^{15}N uptake under drought. Usually, EM root tips contained similar or higher amounts of ^{15}N than NM root tips and only in few cases, N turnover was higher in NM root tips than in EM ones. In particular, we observed that shade and the combination shade with drought induced a shift between NM and EM root tips, for N uptake and turnover. We suggest that C supply to EM fungi is essential for maintaining fungal effectiveness

for N uptake, but the relationship plant C supply-fungal N uptake appears to be very complex, under the plant control, as well as the external factors such as soil microorganisms, including EM fungi, activities.

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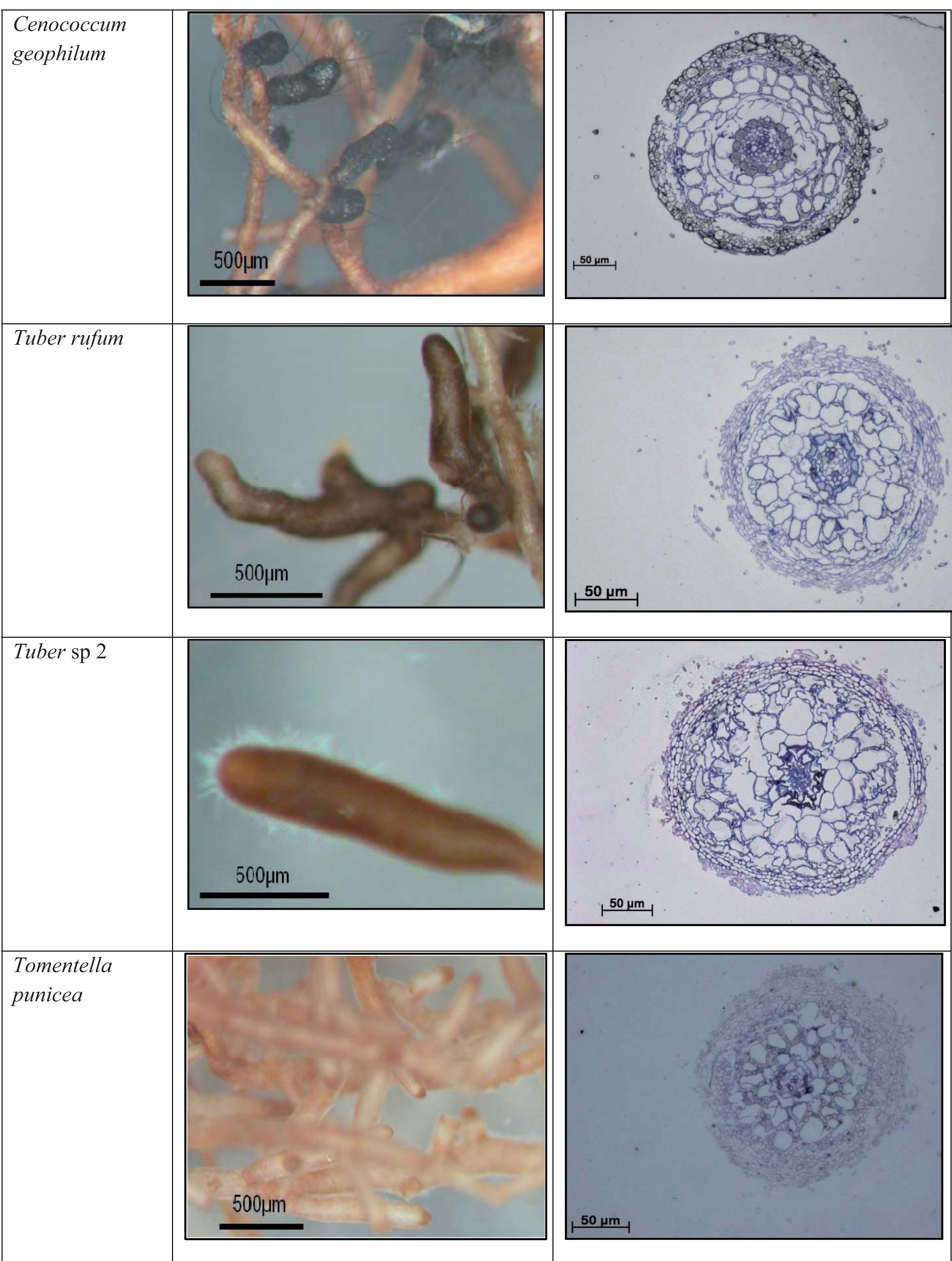
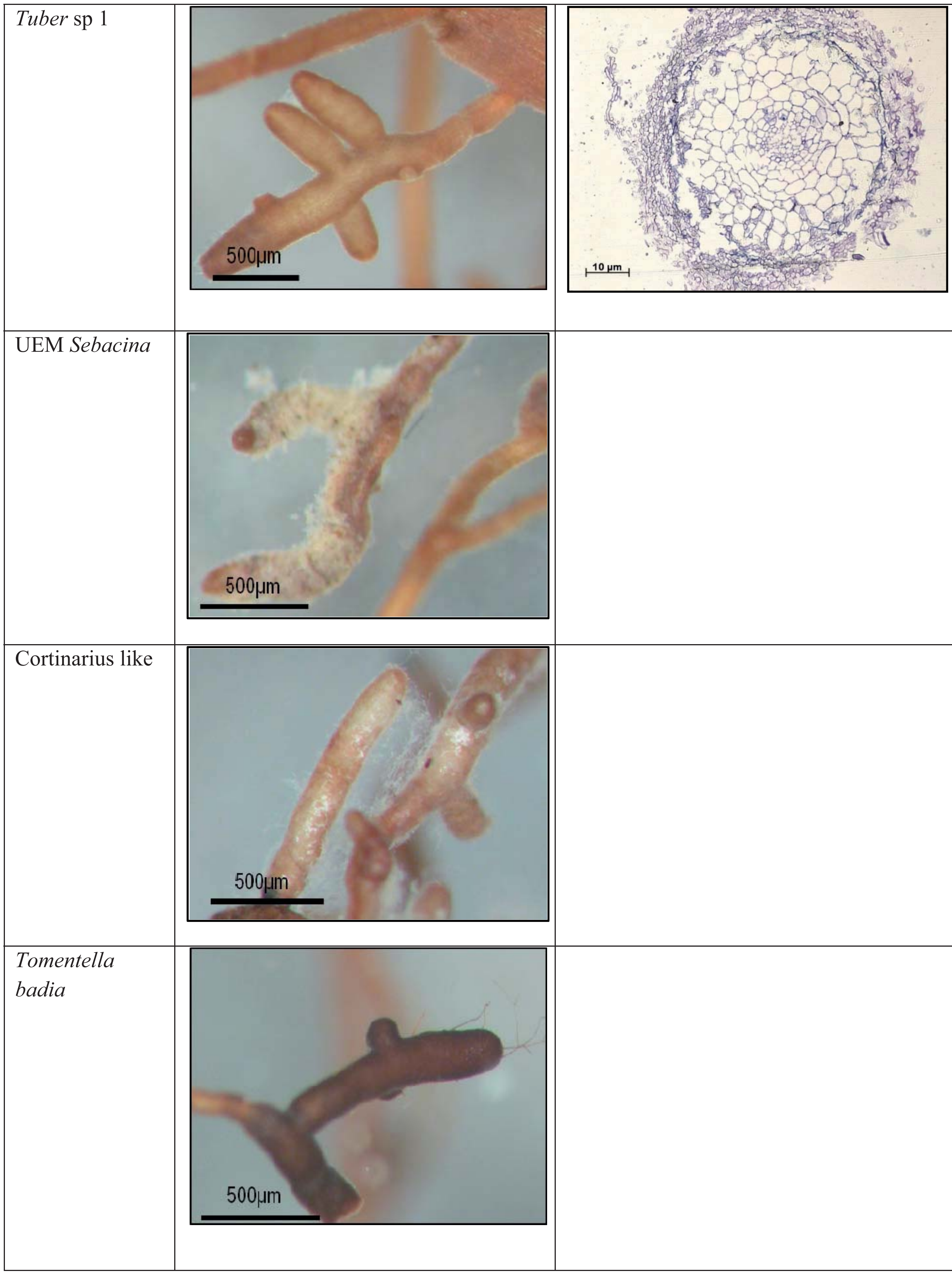
Supplement 3.1

Root tips of young beech plants colonized by different ectomycorrhizal fungi. Stereomicroscope view (left) and cross sections stained with toluidine blue (right).




Tuber sp 1
500µm
10 µm
UEM Sebacina
500µm
Cortinarius like
500µm
Tomentella badia
500µm

UEM Telephoraceae		
Tomentella viridula		

CHAPTER 4: Effects of ectomycorrhizal symbiosis on the nitrogen uptake capacities of young beech (*Fagus sylvatica*) seedlings under drought conditions, influenced by irradiance

4.1. Introduction

Temperate forest ecosystems are characterized by growth-limiting availability of nitrogen (N) (Rennenberg et al. 2009). Most temperate forest trees develop a mutualistic relationship of roots with ectomycorrhizal (EM) soil fungi (Smith and Read 2008). The major function of the mycobiont of EM roots is to facilitate the capture of mineral nutrients present in soil, by extending the absorbing system in the form of an external mycelia network (Tuomi et al. 2001, Gobert and Plassard 2008). The external mycelium play an essential role in the nutrient acquisition process (Kreuzwieser and Gessler 2010) by increasing the soil volume from which nutrients can be absorbed and by achieving into the soil not accessible to roots (Finlay and Read 1986, Finlay et al. 1988, Read and Perez-Moreno 2003). For example, Hobbie and Hobbie (2006) have estimated that 61-86% of the plant N is provided via EM fungi. It has been suggested that natural selection favored appearance of EM mutualism in nutrient limiting environments just because of fungal improvement of plant access to nutrients (Read 1991).

EM fungi are able to take up and assimilate N in mineral forms as ammonium (NH_4^+) and nitrate (NO_3^-), or as organic compounds such as small peptides and amino acids (Smith and Read 2008).

In most forest soils, ammonium is typically the dominant form of inorganic N (Vitousek et al. 1982, Nadelhoffer et al.1984, Marchner and Dell 1994, Gessler et al. 1998a, Usman et al. 2000, Jerabkova et al. 2006). Therefore, a preference of trees for ammonium uptake compared with nitrate is not surprising (Nadelhoffer et al.1984, Plassard et al. 1991, Kronzucker et al. 1995, Högberg et al. 1998, Gessler et al. 1998a, Min et al. 2000). Since the roots are ensheathed by EM fungal mantle, this uptake is mainly achieved by the fungal partner. In fact, intact EM systems use predominantly ammonium (Finlay et al. 1989, Ek et al. 1994, Eltrop and Marschner 1996, Wallander et al. 1997, Javelle et al. 1999, Wu et al. 1999).

The inorganic N uptake depends on one hand on the availability of N at the root surface, which is determined by soil physical and chemical properties e.g. ammonium to nitrate ratio, by ions diffusion or mass flow (Nadelhoffer et al. 1985, BassiriRad et al. 1999). And on the other hand, it is strongly influenced by root architecture (Lynch 1995, Pregitzer et al. 2002, Boukcim and Plassard 2003, Cruz et al. 2004, Helmisaari et al. 2009) and by the physiological efficiency of the root uptake systems (Chapin 1980, Schenk 1996, Gessler et al. 1998a, Glass et al. 2002, Gessler et al. 2005, Högberg et al. 2008).

Root architecture is strongly influenced by the presence of EM symbiosis (Hetrick 1991, Dexheimer and Pargney 1991, Ditengou et al. 2000, Boukcim et al. 2001, Pregitzer et al. 2002, Felten et al. 2009). Interaction with EM fungi involves dramatic changes in root morphology such as stimulation of lateral root formation, increasing of roots lengths, radial elongation of epidermal cells and suppression of root hair development (Ditengou et al. 2000, Boukcim and Plassard 2003, Wu et al. 2009, Felten et al. 2009). The short, lateral fine root branches represent most of the absorbing root surface of forest trees (Pregitzer et al. 1997) and exhibit higher N concentrations and metabolic rates than other parts of the root system (Pregitzer et al. 2002, Helmisaari et al. 2007, Helmisaari et al. 2009).

Formation and functionality of EM symbiosis is based on plant capacity to supply C to associated fungi (Smith and Read 2008). The transfer of current assimilates to soil microorganisms has been estimated using different approaches such short pulse ^{13}C labelling (Högberg et al. 2008, Rühr et al., 2009), concomitant measurements of ^{13}C isotopic signature of CO_2 respired and newly assimilated organic matters pools (Gessler et al. 2007, Kodama et al. 2008), and different experiments that interrupt the photosynthate flux e.g. tree girdling (Högberg et al. 2001, Högberg et al. 2002, Bhupinderpal-Singh et al. 2003. Pena et al. 2010) or free air carbon dioxide enrichment (FACE), which used different isotopic signature to identify new tree derived soil C (Godbold et al. 2006). All these studies have shown that the recent assimilates are rapidly translocated to the belowground compartment and much of this C flux is made available to EM fungi. Field-scale girdling experiment in conifer forest (Högberg et al. 2001) showed that soil microbial respiration decreased in two weeks by 56%, of which 41% was to the loss of EM mycelia, because of an interrupted C flux (Högberg et al. 2001, Högberg et al. 2002). In another study, the contribution of EM external mycelium and roots to forest soil CO_2 efflux has been estimated to about 25 % and about 15%, respectively (Heinemeyer et al. 2007). Furthermore, the FACE experiment in a poplar plantation indicated that EM fungal C constitutes the dominant (62%) C input to soil organic matter (Godbold et al. 2006). Controlled experiments using ^{13}C isotope signatures indicated that as much as 22% of photosynthate was allocated to EM fungi (Hobbie and Hobbie 2006). These examples emphasise the C demand of EM mutualism.

In forest ecosystems, shading by canopy and neighborhood vegetation induces a diminution of photosynthetically active radiation resulting in a reduction in net photosynthesis. Shade tolerant plants, such as beech (Ellenberg and Strutt 2009), have generally lower photosynthetic rates and may allocate less biomass to roots compared with less shade tolerant plants, generating lower carbohydrate availability for EM symbiosis. These differences in plant physiology may result in

differences in EM community structure (Gardes and Bruns 1996; Horton and Bruns 1998). However, the shade effects on EM colonization rate are ranging from no effect (Dehlin et al.2004, Brearly et al. 2007) to strongly diminished (Igleby et al. 1998, Tennakoon et al. 2005, Turner et al. 2009). Druebert et al. (2009) have found for European beech a reduction from 74% EM colonization of plants grown in full light to 10% for plants grown in deep, growth-limiting shade.

Photosynthesis rates decrease under drought by stomatal closure and because of inhibition of Rubisco synthase (Tezara et al. 1999, Cochard et al. 1999, Raftoyannis and Radoglou 2002, Galleand and Feller 2007, Winkler et al. 2010). Water deficits also strongly decrease and delay the downward transport of recent photosynthates (Hölttä et al. 2009, Kuzyakov and Gavrichkova 2010). Ruehr et al. (2009) has evaluated the effects of drought on rates of C flux within young beech trees, by pulse ^{13}C labeling in a microcosms study. They found that, within two days after labeling, drought decreased the contribution of new assimilates to soil respiration by 33%, while the residence time of recent C in leaves increased from 2.4 to 5 days.

Water limitation affects both partners of EM mutualism. EM colonization rate may decrease under low water availability (Nilsen et al. 1998, Swaty et al. 2004) and EM community structure depends on soil moisture content (Gehring et al. 1998). Several studies have shown that EM fungi can improve plant performance under drought (Duddridge et al 1980, Dixon et al. 1983, Brownlee et al. 1983, Davis et al. 1996, Morte et al. 2001, Kennedy and Peay 2007). Severe drought will also affect the fungi and, hence improvements of plant performances are only provided at intermediate levels of soil moisture (Theodorou 1978, Coleman et al. 1989, Dosskey et al. 1991, Kennedy and Peay 2007).

In Central Europe, *Fagus sylvatica* dominates the natural forest vegetation (Ellenberg and Strutt 2009). In the understory of mature beech forest, the seedlings grow under very low light intensity (less than 5% of canopy radiation, Ehrhardt 1988, quoted in Gansert and Sprick 1998). It has been shown that European beech develops physiological strategies to survive under limited light (Gansert and Sprick 1998). When increasing levels of irradiance are available, e.g. in forest gaps the growth of young beech plants is stimulated (Tognetti et al. 1994, Johnson et al. 1997, Tognetti et al. 1998, Collet et al. 2001, Aranda et al. 2004, Parelle et al. 2006). The roots of *Fagus sylvatica* form exclusively EM symbioses and under natural conditions, they are colonized by almost 100% (Rumberger et al. 2004, Buee et al. 2005, Pena et al 2010, Lang et al. 2011). Compared with other temperate broad-leaved species, European beech is more sensitive to drought (Ellenberg and Strutt 2009). Its performance, growth and competitive ability decreased strongly under drought conditions (Peuke et al. 2002, Geßler et al. 2007). The benefit of EM fungi on plant N nutrition under drought conditions may help beech regeneration, since N is a growth-limiting factor and actual climate

scenarios predict frequent drought periods (IPCC 2001). Therefore, a better understanding of EM symbiosis functionality with respect to N uptake under different environmental conditions such shade and drought is necessary.

In the present study, we aimed to answer at three questions: (1) Do EM exhibit positive effects on growth, N uptake and N allocation in young beech plants grown under drought conditions? (2) Are the functional capacities of EM fungi affected by limited plant carbon productivity by shading? (3) Do influence the differences in root architecture the N uptake under limited water availability?

To address these questions, we exposed young beech plants, either whose root systems were colonized by typical EM fungal communities, or which were non-mycorrhizal (NM), to light and shade, respectively and stressed the plants by limiting water availability. Before harvest, the plants received labeled nitrogen (^{15}N) to determine N uptake. Growth performance, root architecture and demography, ^{15}N uptake and partitioning between root tips, roots, stems and leaves, total N and C concentrations in above and belowground biomass of EM and NM plants were measured.

4.2. Materials and methods

4.2.1. Experimental design

Beech nuts (provenance: Forstsaatgutstelle Oerrel, Niedersachsen, Germany) were germinated on moist filter paper at 4°C, in darkness for four weeks. When the radicles had reached a length of 1-2 cm, the seed coats were removed and the seedlings were sterilized in a solution of 1 ml fungicide Proplant (Stähler, Stade, Germany) and 100 mg tetracycline (Duchefa Biochemie, Haarlem, Holland) in 1 l distilled water for 24 hours at room temperature. Afterwards, they were rinsed three times with tap water. Sterilized seedlings were planted in 1 l pots filled with forest soil and transferred to the greenhouse.

The soil was obtained from a mature beech stand in Tuttlingen forest (latitude 47°59`N, longitude 8°45`E, see Dannenmann et al. 2009 for more details) by collecting the Ah horizon (20 cm depth). The soil was sieved through a mesh (1 cm width). Half of the soil (O) was kept in darkness at 4°C until planting. The other half (S) was sterilized at 121°C and 0.11 MPa (HST 6x6x6, Zirbus Technology, Bad Grund, Germany) once a week three times. The pH values of the sterilized and the original soil were 6.46 (±0.07) and 7.02 (±0.02), respectively. Soil chemical composition was unaltered (Table 4.1).

Table 4.1: Soil pH and mineral elements (mg g^{-1} dry wt). *P* value for the t-test comparing sterilized and original soil.

	Sterile soil	Original soil	*P* value
pH (H_2O)	6.46 ± 0.07	7.02 ± 0.02	< 0.001
C	96.76 ± 1.96	102.2 ± 2.40	0.048
N	5.74 ± 0.19	5.96 ± 0.14	0.176
C/N	16.87 ± 0.41	17.20 ± 0.54	0.135
P	0.77 ± 0.02	0.80 ± 0.01	0.355
K	10.90 ± 0.29	10.61 ± 0.23	0.210
Ca	12.43 ± 0.21	12.73 ± 0.12	0.210
Mg	7.41 ± 0.21	7.29 ± 0.13	0.298
Mn	0.99 ± 0.02	0.92 ± 0.02	0.019
Mg	3.03 ± 0.08	3.00 ± 0.05	0.364

SE = standard error of means (n=5)

After planting the seedlings into original or sterile soil, they were grown in a greenhouse under ambient conditions and watered regularly with tap water. After 4 months, when the seedlings in original soil had developed EM roots, the young beech plants were transferred individually to 660 ml pots filled with a mixture of fine sand (0.4-0.8 mm), gross sand (0.7-1.2 mm) and peat in a proportion of 4:5:1. The pots were placed in a greenhouse cabinet and maintained at: 16 h day length, 20°C temperature, and 55% relative air humidity. The EM and NM beech plants were exposed to two light levels: (L) full light, with a photosynthetic active radiation (PAR) of 200 µmol m^{-2} s^{-1} (light fittings for greenhouse, series 3071, Schuch, Worms, Germany), and (S) low irradiance with 35-40 µmol PAR m^{-2} s^{-1} at plant height obtained by installing a green double layer polyethylene shading net (5x5 mm mesh Mayer, Rellingen, Germany) above the seedlings. The irradiance levels were measured using a quantum photometer Li-185B with a quantum sensor Li-190SB (LiCor INC., Lincoln, USA). Each plant received eight ml of a Hoagland-based nutrient solution after Dyckmans and Flessa (2001) containing 0.4 mM NH_4Cl, 0.05 mM $NaSO_4$, 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 $MnSO_4$, 0.005 mM $FeCl_3$, 0.15 µM $ZnCl_2$, 0.1 µM MoO_3, 0.064 µM $CuCl_2$ (pH 3.9) seven times a day. The only nitrogen source was ammonium. Soil

moisture content was measured with ThetaProbe ML2X soil moisture sensor equipped with a HH2 readout unit (Delta-T Devices Ltd, Cambridge, UK) and was maintained above 0.040 m^3m^{-3}.
After 2 months in the sand culture, half of the seedlings from each growth regime were subjected to drought stress. The volume of watering solution was diminished by 62.5 % to 3 ml per plant and irrigation event. Thereby, the soil moisture content decreased below 0.035 m^3m^{-3}. The drought treatment lasted for 16 days. During this time, leaf predawn water potential (Ψ_{pd}) was measured regularly, using a Scholander pressure chamber (Soil-moisture Equipment Corp., Santa Barbara, CA). ^{15}N labelling was applied for last three days before harvest. NH_4Cl in the nutrient solution was replaced by 2 mM $^{15}NH_4Cl$ (99 atom %) (Cambridge Isotope Laboratories, Inc, Hampshire, GB). Each seedling received 1.864 mg ^{15}N during the time course of 3 days. All seedlings (control and drought) were supplied with the same amount of nutrients (21 ml per day). The control plants were irrigated additionally 7 times a day with 5 ml deionized water in the intervals between nutrient solution applications.
Each growth regimes comprised twenty-four ^{15}N labeled and three non-labeled plants.

4.2.2. Harvest

The plants were harvested after 72 h of ^{15}N labelling. Roots were briefly rinsed with deionised water to remove adhering particles and nutrients. Leaves, stems and roots were separated and the biomass was determined for each component. Except the roots, which were further used for mycorrhizal analysis the remaining materials were dried at 60°C and used for measurement of dry mass, C, N and ^{15}N concentrations. Roots were kept at 4°C in plastic bags until morphotyping.

4.2.3. Collection of root tips for isotope analysis

Whole root systems of ten seedlings per treatment were analyzed. Each root tip was examined under a stereomicroscope (Leica M205 FA, Wetzlar, Germany) and assigned to one of the following root fractions: vital ectomycorrhizal (EM), vital non-ectomycorrhizal (NM), dead ectomycorrhizal (Dry-EM) and dead non-ectomycorrhizal (Dry-NM). Live and dead roots were separated according to Winkler et al. (2010). Vital and dead ectomycorrhizal root tips were sorted as described by Downes (1992). A total of twenty to sixty tips were required for one sample suitable for isotope measurement. EM morphotypes and other classes of root tips of each plant were cut under the compound microscope. Two different sets of instruments were used to handle non-labeled and labeled plants to avoid cross contamination. EM tips were excised at the level of last lateral root ramification,

ensheathed by the mantel; NM tips were sampled at the level of the youngest and active "white" zone (Enstone et al 2001; Evert and Eichhorn 2007); Dry-EM tips were cut at the same position as NM tips. Samples were freeze dried for isotope analysis.

4.2.4. Analysis of root architecture

The root systems were analyzed by measuring root lengths and surface areas using a commercially available root analysis software package (WinRHIZO V3.10b; Regent Instruments Inc., Quebec, Canada). The root systems were scanned for image acquisition using a scanner (HP 4Jc, Hewlett-Packard, Dübendorf, Switzerland). For proper analysis, separation of fine and coarse roots was necessary. By comparison with results from stereomicroscopy, two software parameters (scanning resolution and image transformation threshold) were found to have a large impact on the analysis of the scanned images. The scanning resolution was set to 600 dpi for fine and 300 dpi for coarse roots, and threshold was selected at "pale roots" with "more sensitivity" for fine and "dark roots" with "less sensitivity" for coarse roots.

4.2.5. Isotope analysis of plant tissues

Dry subsamples of fine roots, stem and leaves were ground with a ball mill (Retch, Hann, Germany) to a fine powder and weighed. Freeze-dried root tips were weighed; 0.2 to 1.1 mg dry mass per sample were necessary for isotope estimation. To determine the mass of root tips 20 dry root tips were weighted per EM species and other root tip classes (5 per treatment), using a super-micro balance (S4, Sartorius, Göttingen, Germany).

Total C, N and ^{15}N concentrations in root tips were determined at the Centre for Stable Isotope Research and Analysis (University Göttingen) with an isotope ratio mass spectrometer (IRMS Delta ^{plus}, Thermo Finnigan Mat, Bremen, Germany) coupled to an elemental analyzer (EA 1108, Fisons, Rodano, Italy). C, N, ^{15}N in milled plant samples (root, stem, leaf) were measured at Institute of Forest Botany and Tree Physiology (University of Freiburg), using a similar elemental analyzer (NA 2500, CE Instruments, Milan, Italy).

Total plant N (mg plant^{-1}) was determined by summing the N content (mg) of all individual plant parts (i.e. root, stem, leaf). Total plant C was calculated similarly.

The relative ^{15}N abundance was expressed as the ratio:

$$^{15}N\ (\text{atom }\%) = \frac{^{15}N}{^{14}N + ^{15}N} \times 100$$

The ^{15}N content for each plant tissues (root tips, roots, stem, and leaves) was calculated as:

$$^{15}N_{tissue}\ (mg) = \frac{(^{15}N_{l} - {}^{15}N_{nl})}{100} \times N_{tissue} \times m_{tissue}$$

where $^{15}N_{l}$ and $^{15}N_{nl}$ refer to atom % concentrations in labelled ($^{15}N_{l}$) and non-labelled plants ($^{15}N_{nl}$); N_{tissue} is concentration of total N in the plant tissue (mg g^{-1}); m_{tissue} is the tissue dry mass (g).
Plant ^{15}N uptake was calculated as the sum of $^{15}N_{roots}$, $^{15}N_{stem}$ and $^{15}N_{leaves}$.

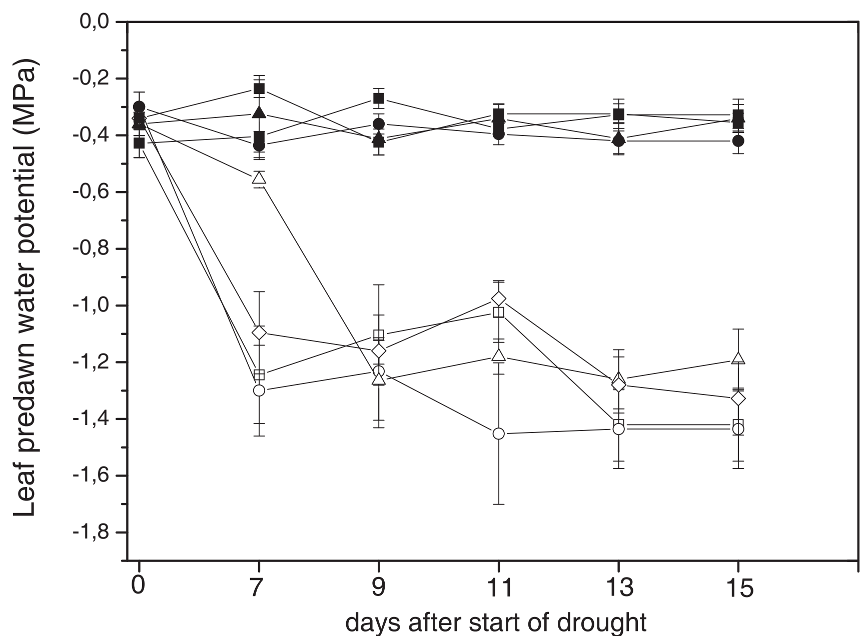
4.2.6. Calculations and statistical analysis

Statistical analysis was performed using Statgraphics Plus 3.0 (StatPoint, Inc., St Louis, MO, USA). Results were compared by one-way and multifactor analysis of variance (ANOVA). The effect of limited water availability combined with presence of EM, light irradiance and time on development of predawn water potential was analyzed by repeated-measures ANOVA by the General Linear Models (GLM). When necessary, data were transformed logarithmically or by square root to satisfy the criteria of normal distribution and homogeneity of variance. When transformation of the data did not meet these requirements, the non-parametric Kruskal-Wallis and Mann-Whitney W tests were applied instead ANOVA. $P \leq 0.05$ was considered significant.
Regression analyses were plotted with the program Origin 7G (OriginLab Corporation, Northampton, MA).

4.3. Results

4.3.1. Physiological response to drought

Predawn leaf water potential (Ψ_{pd}) decreased significantly seven days after the start of diminished water supply, with the exception of EM plants grown under shade (Figure 4.1). Plants with about 39% of root tips colonized by EM fungi displayed a slower reduction of Ψ_{pd}, reaching a Ψ_{pd} of about -1.10 MPa two days later. Repeated-measures ANOVA revealed a strong influence of the presence of EM roots on Ψ_{pd} ($P \leq 0.001$, $F = 9.51$) and a significant effect of full light versus shade ($P = 0.027$, $F = 4.95$). The diminished water supply resulted in all treatments in a mean Ψ_{pd} of -1.34 ± 0.12 MPa between 9 and 16 days (Figure 4.1).



	F-ratio	*P*-value
EM	9.5	**< 0.001**
Light	4.9	**0.027**
Water	212.9	**< 0.001**
Time	11.8	**< 0.001**

Figure 4.1: Leaf predawn water potential in irrigated (close symbols) and drought stressed (open symbols) beech (*Fagus sylvatica*) plants. Ectomycorrhizal plants grown under full light (squares), ectomycorrhizal plants grown under shade (triangles), non-ectomycorrhizal plants grown under full irradiance (circles), non-ectomycorrhizal plants grown under shade (diamonds). Bars indicate means ± SE of 5 measurements per treatment and time point. F and P values obtained by repeated-measures analysis of variance are shown next to the figure.

4.3.3. Growth parameters

No significant effects of drought (P = 0.217, F = 1.58), or presence of EM fungi (P = 0.162, F = 2.04), were found on whole plant dry biomass (Table 4.3). The plants grown under shade had a reduced biomass (P = 0.049, F = 4.15), compared with those grown under full light (Table 4.3). The presence of EM fungi significantly increased the biomass of leaves (P = 0.007, F = 8.08, Table 3).
The SLA was significantly higher in EM plants compared with NM ones (P < 0.001, F = 938.09) and for the plants grown in shade compared with those grown under full light (P = 0.002, F= 10.33, Table 4.3). Drought had no significant effect on the specific leaf area (SLA, m^2 g^{-1}) of EM or NM beech plants.

A significant effect of irradiance on the root-to-shoot ratio was found both for EM and NM plants (P =0.001, F= 22.11). In full light the root-to-shoot ratio was higher due significant higher mass of roots than under shade (P =0.010, F=7.48, Table 4.3). The presence of EM had no significant effects on root- to-shoot ratios (P =0.384. F = 0.78, Table 4.3).

4.3.4. ^{15}N uptake and partitioning

The presence of EM fungi increased ^{15}N uptake per plant (P = 0.041, F = 4.49, Figure 4.2B). However, such an effect was not observed for the specific ^{15}N uptake of beech plants (P = 0.176, F = 1.91), excepting the plants grown in full light with water limitation (P = 0.047, F = 2.46, Figure 4.2A). Plants grown in shade showed significantly lower specific ^{15}N uptake (P < 0.001, F = 15.43, Figure 4.2A) and ^{15}N uptake per plant (P < 0.001, F = 25.23, Figure 4.2B) compared with those from full light.

The internal partitioning of ^{15}N taken up by EM and NM beech plants grown under different levels of water and irradiance is shown in Table 4.4. In all treatments, the highest ^{15}N fractions, about 60 to 80%, were present in roots. Under shade ^{15}N allocation to leaves significantly increased (P < 0.001, F = 43.81, Table 4.4). Presence of EM fungi significantly increased the ^{15}N allocation to leaves compare with those of NM plants (P = 0.026, F = 5.43, Table 4.4). Partitioning of ^{15}N between roots, stem and leaf tissues was not significantly influenced by drought (Table 4.4).

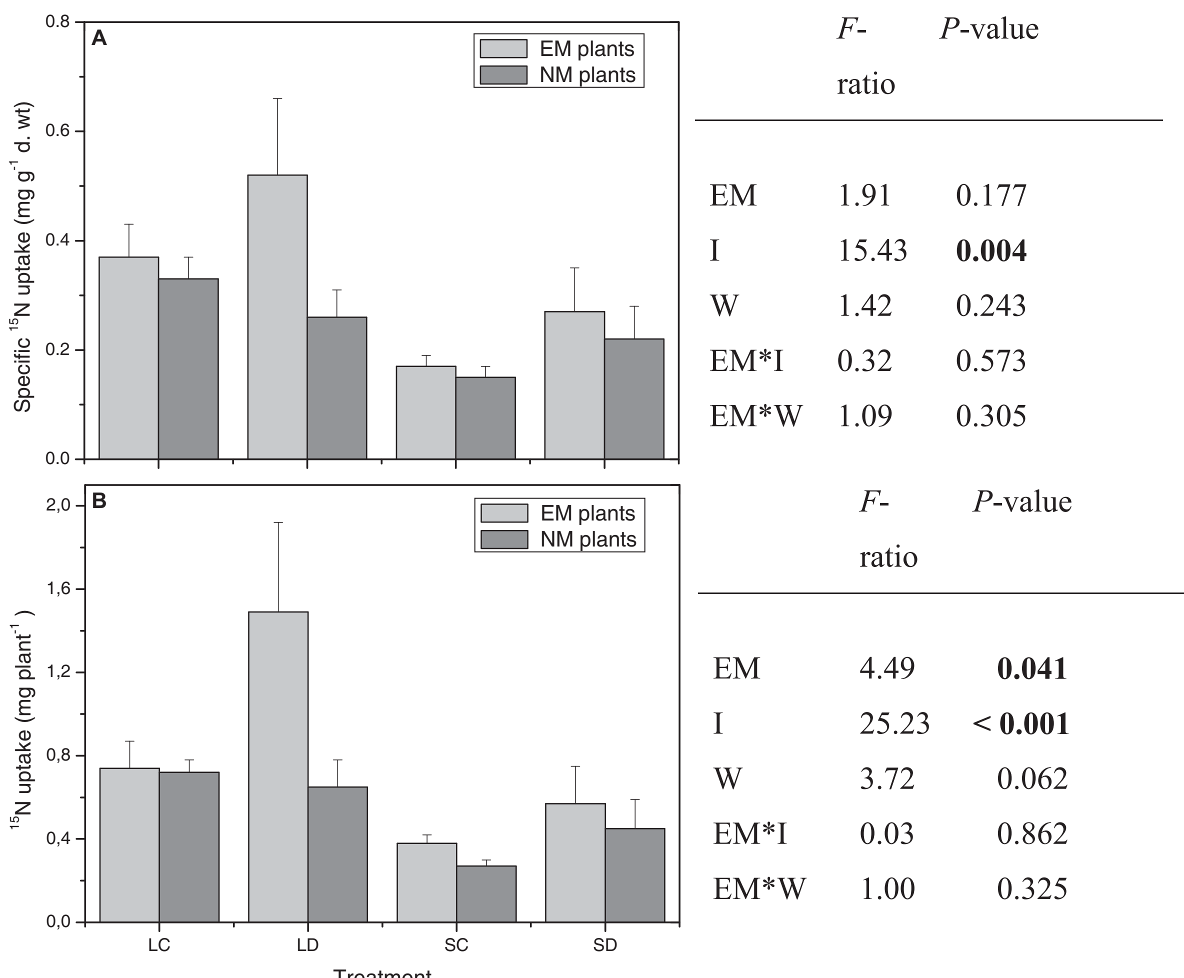
Table 4.2: Root morphology and demography characterized by total length, surface area, number of root tips and the percentages represented by non-myccorhizal (NM), ectomycorrhizal (EM), dry non-mycorrhizal (Dry-NM) and dry ectomycorrhizal (Dry-EM) root tips under two levels of irradiance and water availability (LC, full light control; LD, full light drought; SC, shade control; SD, shade drought). Mean values ± SE (n = 10, n = 5 for NM plants in LC and LD treatments). Below: statistical effects of ectomycorrhizas presence (EM), irradiance (I) and water availability (W) on root length and surface, total number of root tips and percentages of different root fractions assessed by ANOVA multi-factors analysis. P ≤ 0.05 are shown by bold letters.

Growth regimes	Root total length (cm)	Root surface area (cm^2)	Number of root tips	Vital root tips (%)	NM root tips (%)	EM root tips (%)	Dry-NM root tips (%)	Dry-EM root tips (%)
EM-LC	2622 ± 305	437 ± 47	5365 ± 675	81 ± 2	51 ± 4	30 ± 7	15 ± 1	4 ± 1
EM-LD	3048 ± 519	511 ± 80	7315 ± 754	80 ± 2	48 ± 3	32 ± 11	15 ± 1	4 ± 1
EM-SC	1643 ± 219	273 ± 35	4943 ± 658	77 ± 2	45 ± 2	31 ± 1	20 ± 2	3 ± 1
EM-SD	1644 ± 188	282 ± 30	5530 ± 722	78 ± 4	39 ± 1	39 ± 3	18 ± 3	4 ± 1
NM-LC	1854 ± 274	339 ± 50	2831 ± 321	89 ± 2	89 ± 3	0 ± 0	11 ± 3	0 ± 0
NM-LD	1832 ± 262	339 ± 46	4644 ± 717	77 ± 5	75 ± 5	0 ± 0	23 ± 5	0 ± 0
NM-SC	948 ± 97	180 ± 17	2524 ± 275	87 ± 2	87 ± 2	0 ± 0	13 ± 2	0 ± 0
NM-SD	1083 ± 98	200 ± 17	2723 ± 251	85 ± 4	85 ± 4	0 ± 0	15 ± 4	0 ± 0

	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
EM	8.42	**0.005**	5.16	**0.026**	28.98	**0.001**	5.91	**0.017**	211.2	<**0.000**	-	-	3.54	0.064	-	-
I	19.36	**0.001**	20.45	**0.001**	6.19	**0.015**	0.03	0.854	0.40	0.527	1.25	0.270	0.03	0.865	0.00	0.985
W	0.25	0.616	0.35	0.555	5.31	**0.024**	1.20	0.277	4.64	0.035	1.61	0.212	0.97	0.329	0.88	0.354

Table 4.3: Effects of the presence of EM fungi (EM, ectomycorrhizal; NM, non-mycorrhizal beech plants), irradiance and water availability (LC, full light control; LD, full light drought; SC, shade control; SD, shade drought) on roots, stem, leaves and whole plant dry biomass, on specific leaf area (SLA) and on plant root-to-shoot ratio. Mean values ± SE (n = 5). Below: statistical effects of ectomycorrhizas presence (EM), irradiance (I) and water availability (W) on roots, stems, leaves and whole plant dry biomass, on specific leaf area (SLA) and on plant root-to-shoot ratio assessed by ANOVA multi-factors analysis. P ≤ 0.05 are shown by bold letters.

Growth regimes	Roots dry biomass (g)		Stem dry biomass (g)		Leaves dry biomass (g)		Plant dry biomass (g)		SLA(m^2g^{-1}) x 10^3		Root-to-shoot ratio	
EM-LC	1.35 ± 0.17		0.46 ± 0.07		0.33 ± 0.03		2.14 ± 0.25		15.90 ± 0.69		1.72 ± 0.07	
EM-LD	1.80 ± 0.14		0.71 ± 0.14		0.43 ± 0.07		2.94 ± 0.40		16.33 ± 0.59		1.63 ± 0.15	
EM-SC	1.47 ± 0.05		0.62 ± 0.05		0.41 ± 0.03		2.39 ± 0.15		17.60 ± 0.36		1.43 ± 0.10	
EM-SD	1.17 ± 0.06		0.65 ± 0.06		0.37 ± 0.04		2.08 ± 0.13		17.91 ± 0.58		1.16 ± 0.15	
NM-LC	1.42 ± 0.05		0.61 ± 0.05		0.25 ± 0.04		2.25 ± 0.27		8.04 ± 0.17		1.65 ± 0.14	
NM-LD	1.64 ± 0.08		0.70 ± 0.08		0.33 ± 0.02		2.58 ± 0.30		7.15 ± 0.27		1.60 ± 0.18	
NM-SC	1.10 ± 0.09		0.57 ± 0.09		0.32 ± 0.03		1.87 ± 0.23		7.93 ± 0.31		1.24 ± 0.04	
NM-SD	1.11 ± 0.04		0.61 ± 0.04		0.33 ± 0.03		2.03 ± 0.16		8.10 ± 0.19		1.18 ± 0.13	
	F	P	F	P	F	P	F	P	F	P	F	P
EM	1.40	0.245	0.03	0.863	8.08	**0.007**	1.58	0.217	938.09	**0.001**	0.78	0.384
I	7.48	**0.010**	0.01	0.978	0.62	0.435	4.15	**0.049**	10.33	**0.002**	22.11	**0.001**
W	0.68	0.414	3.75	0.061	2.20	0.147	2.04	0.162	0.01	0.917	2.08	0.150



	F-ratio	*P*-value
EM	1.91	0.177
I	15.43	**0.004**
W	1.42	0.243
EM*I	0.32	0.573
EM*W	1.09	0.305

	F-ratio	*P*-value
EM	4.49	**0.041**
I	25.23	**< 0.001**
W	3.72	0.062
EM*I	0.03	0.862
EM*W	1.00	0.325

Figure 4.2: Effect of the presence of ectomycorrhizal fungi (EM, ectomycorrhizal plants; NM, non mycorrhizal plants), light irradiance and water availability (LC, full light control; LD, full light drought; SC, shade control; SD, shade drought) on the ^{15}N concentration (A) and ^{15}N content (B) of whole beech plants. All plants were supplied with 1.864 mg ^{15}N during a 3-day labeling period. Bars indicate mean values ± SE (n=5). Right side: statistical effects of ectomycorrhizas presence (EM), irradiance (I) and water availability (W) on ^{15}N uptake assessed by ANOVA multi-factors analysis. P-values ≤ 0.05 are shown by bold.

4.3.5. Total nitrogen and carbon concentrations

N concentrations in stem and leaves were significantly lower in EM plants than in NM plants ($P < 0.001$, $F = 28.31$; $P = 0.001$, $F = 19.19$, Table 4.5). While in the roots no significant effect of EM fungal presence, irradiance or availability of water was found. However, the EM presence had a significant effect at the whole plant level on N concentrations ($P = 0.009$, $F = 7.59$, Table 4.5). EM plants showed lower total N concentrations than NM plants (Table 4.5). Irradiance and drought had no significant effect on the N concentrations in beech plants.

Table 4.5: Nitrogen concentration (mg g^{-1} d. wt) of roots, stem and leaves on a mass basis of ectomycorrhizal (EM) and non-mycorrhizal (NM) beech plants, under two levels of irradiance and water availability (LC, full light control; LD, full light drought; SC, shade control; SD, shade drought). Mean values ± SE (n = 5). Below: statistical significant effect of ectomycorrhizas presence (EM), irradiance (I) and water availability (W) on total nitrogen concentration (mg g^{-1} d. wt) of roots, stems and leaves on a mass basis assessed by ANOVA multi-factors analysis. P ≤ 0.05 are shown by bold letters.

Growth regimes	Roots (mg g^{-1})		Stem (mg g^{-1})		Leaves (mg g^{-1})		Whole plant (mg g^{-1})	
EM-LC	13.77 ± 0.81		12.17 ± 0.87		14.26 ± 0.64		13.51 ± 0.74	
EM-LD	12.46 ± 1.17		7.85 ± 0.85		13.47 ± 0.75		11.50 ± 0.94	
EM-SC	14.48 ± 0.64		8.84 ± 0.61		18.45 ± 0.44		14.31 ± 0.43	
EM-SD	11.26 ± 3.22		10.26 ± 1.05		17.15 ± 0.72		14.61 ± 0.45	
NM-LC	16.75 ± 1.73		15.03 ± 1.36		19.00 ± 0.68		16.78 ± 1.44	
NM-LD	15.09 ± 0.86		12.62 ± 1.15		16.67 ± 0.68		15.24 ± 1.09	
NM-SC	13.69 ± 0.50		12.82 ± 0.82		17.97 ± 0.65		15.06 ± 0.60	
NM-SD	13.46 ± 0.71		12.11 ± 1.49		19.03 ± 0.66		14.01 ± 0.88	
	F	P	F	P	F	P	F	P
EM	0.80	0.376	28.31	**< 0.001**	19.19	**0.001**	7.59	**0.009**
I	0.57	0.455	1.78	0.190	20.94	**0.001**	0.15	0.700
W	1.77	0.192	4.46	**0.042**	1.81	0.188	3.11	0.087

C concentrations on the level of total mass of EM and NM plants did not vary with irradiance or water availability (Table 4.6). EM plants had significantly higher C concentrations in leaves (P = 0.007, F = 8.13) compared with NM plants (Table 4.6). Furthermore, the leaves of beech plants grown under full light had higher C concentrations than those grown under shade (P = 0.012, F = 7.06, Table 4.6).

Table 4.6: Total carbon concentration (mg g^{-1} d. wt) of roots, stems and leaves on a mass basis of ectomycorrhizal (EM) and non-mycorrhizal (NM) beech plants, under two levels of irradiance and water availability (LC, full light control; LD, full light drought; SC, shade control; SD, shade drought). Mean values ± SE (n = 5). Below: statistical significant effect of ectomycorrhizas presence (EM), irradiance (I) and water availability (W) on total carbon concentration of roots, stems and leaves on a mass basis assessed by ANOVA multi-factors analysis. P ≤ 0.05 are shown by bold letters.

Growth regimes	Roots (mg g^{-1})		Stem (mg g^{-1})		Leaves (mg g^{-1})		Whole plant (mg g^{-1})	
EM-LC	527 ± 1		432 ± 6		465 ± 2		497 ± 2	
EM-LD	522 ± 8		438 ± 2		467 ± 2		496 ± 8	
EM-SC	529 ± 14		446 ± 3		457 ± 2		518 ± 14	
EM-SD	514 ± 8		446 ± 5		461 ± 2		517 ± 19	
NM-LC	519 ± 14		450 ± 15		460 ± 5		502 ± 14	
NM-LD	530 ± 10		419 ± 13		455 ± 2		508 ± 7	
NM-SC	530 ± 3		445 ± 3		451 ± 6		525 ± 13	
NM-SD	524 ± 23		447 ± 5		454 ± 2		492 ± 24	
	F	P	F	P	F	P	F	P
EM	0.08	0.785	0.33	0.570	8.13	**0.007**	0.01	0.969
I	0.01	0.983	4.77	**0.036**	7.06	**0.012**	0.15	0.281
W	0.30	0.586	0.59	0.446	0.19	0.669	3.11	0.409

4.3.6. ^{15}N, total nitrogen and total carbon amounts in root tips

In addition to the partitioning of ^{15}N, N and C between roots, stems, and leaves we also measured these elements in root tips. For this propose all root tips were counted and separated in different categories according with vitality, vital and dead root tips, and EM colonization, non-colonized and EM colonized root tips. The letter category was further divided with fungal species. ^{15}N, total nitrogen and total carbon were analyzed in all these root tips categories and presented in the Chapter 3. Here we show the values obtained in the total number of root tips of EM and NM plants.

The ^{15}N amount found in the root tips of beech plants was strongly influenced by the presence of EM, as well as by irradiance and drought (Table 4.7). EM plants showed higher ^{15}N amounts than NM plants (P < 0.001, F = 44.61, Table 4.7). Both shade and drought decreased the ^{15}N amounts in EM or NM beech plants (irradiance P < 0.001, F = 78.09; water availability P = 0.003, F = 8.97, Table 4.7). N and C amounts in the root tips tissues were strongly influenced by the presence of EM fungi (total nitrogen P < 0.001, F = 31.52; total carbon P< 0.001, F = 23.73). The EM plants

displayed higher amounts of total N and total C at the level of the root tips, than NM plants (Table 4.7). This result was obtained mainly because of the significantly higher number of root tips of EM plants than those of NM plants (Table 4.2). Restriction of irradiance decreased significantly the total N amount in the root tips ($P = 0.024$, $F = 5.31$, Table 4.7).

Table 4.7: The amount of ^{15}N, N and C in the sum of root tips per ectomycorrhizal (EM) and non-mycorrhizal (NM) beech plants (µg per sum of plant root tips), under two levels of irradiance and water availability (LC, full light control; LD, full light drought; SC, shade control; SD, shade drought). Mean values ± SE (n = 10, n = 5 for NM plants in LC and LD treatments). Below: statistical effects of ectomycorrhizas presence (EM), irradiance (I) and water availability (W) on the amount of ^{15}N, N and C in the sum of root tips assessed by ANOVA multi-factors analysis. $P \leq 0.05$ are shown by bold letters.

Growth regimes	^{15}N in root tips (µg plant^{-1})	N in root tips (µg plant^{-1})	C in root tips (µg plant^{-1})
EM-LC	37.52 ± 5.32	1018 ± 140	13907 ± 1889
EM-LD	36.19 ± 5.68	976 ± 98	16625 ± 1697
EM-SC	16.98 ± 2.33	800 ±119	12589 ± 1894
EM-SD	12.35 ± 2.43	759 ± 123	12082 ± 1875
NM-LC	25.37 ± 3.47	479 ±65	7192 ± 983
NM-LD	12.32 ± 2.35	430 ± 82	7608 ±1454
NM-SC	6.60 ± 0.83	400 ± 50	6634 ± 838
NM-SD	4.01 ± 0.48	375 ± 45	7001 ± 841

	^{15}N		N		C	
	F	P	F	P	F	P
EM	44.61	**< 0.001**	31.52	**< 0.001**	23.73	**< 0.001**
I	78.09	**< 0.001**	5.31	**0.024**	3.39	0.070
W	8.97	**0.003**	0.10	0.755	0.60	0.440

4.3.7. Correlation between different fractions of nitrogen (^{15}N and total N) in root tips and plant belowground and aboveground biomass

To find out whether ^{15}N and total N amounts found in the root tips of beech plants corresponded to the ^{15}N and total N accumulation in roots or aboveground plant biomass, correlation coefficients were calculated. We found positive correlations between ^{15}N amounts located in the root tips and

those in aboveground plant biomass (r = 0.40, P = 0.014, Figure 4.3A), as well as between ^{15}N amounts in the root tips and in belowground plant biomass (r_s = 0.44, P = 0.016, Figure 4.3B). However, the content of total N in the root tips was negatively correlated with total N found in the aboveground plant biomass (r = - 0.47, P = 0.036, Figure 4.3C), and it was not correlated with the total N in the aboveground plant biomass (r = - 0.09, P = 0.568, Figure 4.3D).

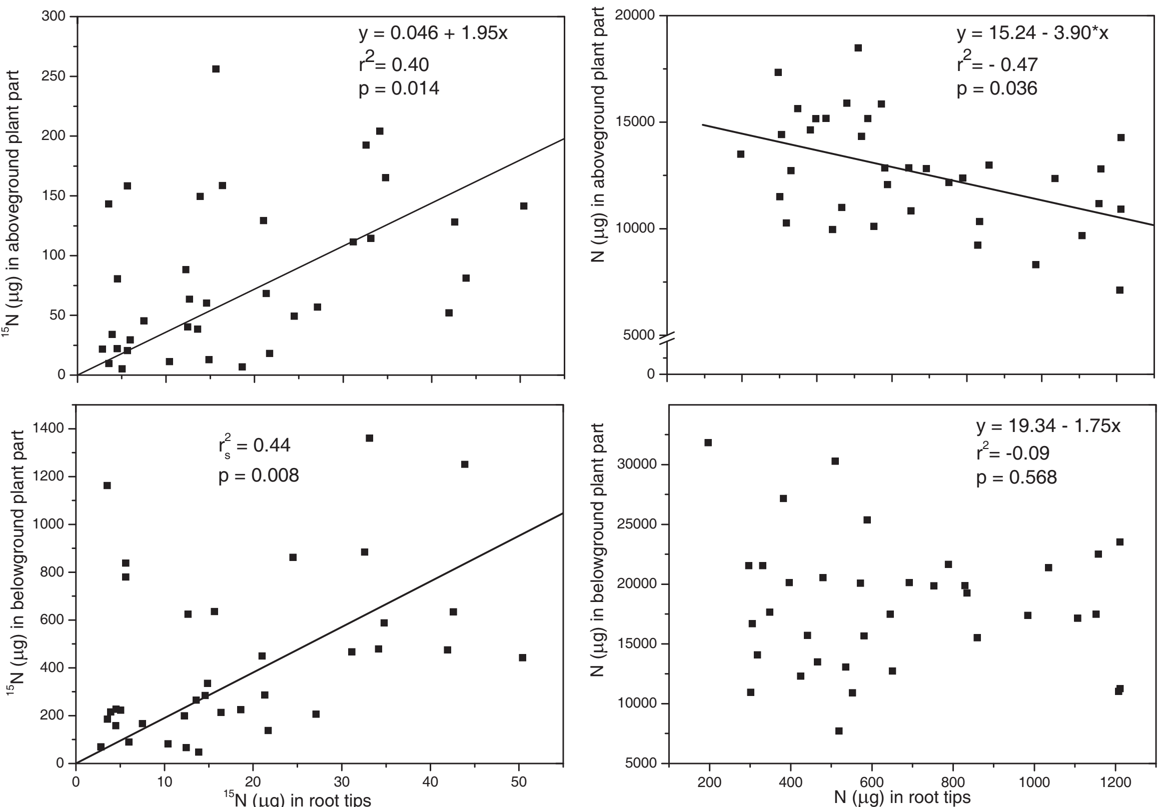


Figure 4.3. Correlation analysis between the amount of ^{15}N in the sum of root tips and the amount of ^{15}N in aboveground plant biomass (stem + leaves) (A), and the amount of ^{15}N in belowground plant biomass (roots – root tips) (B). Correlation analysis between the amount of N in the sum of root tips and the amount of N in aboveground plant biomass (stem + leaves) (C), and the amount of N in belowground plant biomass (roots – root tips) (D).

4.4. Discussion

In the present study, we investigated the effects of irradiance on abilities of EM compared with NM roots to improve growth and N nutrition of young beech plants under drought.

The young beech plants used in our experiment had about 30 % EM root tips. The EM colonization rate is usually much higher for mature beech trees (Rumberger et al. 2004, Buee et al. 2005), but this lower rates have typically been found for few-months-old broad-leaved trees in the field (Lewis et al.

2008). In our experiment, EM fungal colonization did not improve the biomass and total N concentration in any treatment. Moreover, the leaf N concentration was significantly higher in NM than in EM plants. These results were contrary to several other studies that involve pine seedlings (Chu-Chou et al. 1985, Plassard et al. 2000, Rincon et al. 2005, Kennedy et al. 2007) and spruce seedlings (Eltrop and Marschner 1996). All these studies compared abilities of NM and EM roots, colonized by independent fungal species or a combination of two (Kennedy et al. 2007) or three fungi (Chu-Chou et al. 1985), to improve N concentrations in leaves, under control growth conditions. Eltrop and Marschner (1996) and Plasssard et al. (2000) investigated comparative efficiency of few EM fungi on plant N uptake from different sources such as ammonia, nitrate and organic. Despite the fact that different EM fungal species showed own preferences for N source supply, all the EM plants supplied with ammonium had higher leaf N concentrations than NM plants. Nonetheless, our results were in agreement with a more recent study (Jones et al. 2009) that reported a negative correlation between N concentrations in shoots with colonization degree. Here we found no positive influence of EM on plant N concentrations, probably because of permanent N availability in the nutrient solution (Correa et al. 2006, Correa et al. 2008). EM fungi stimulate N uptake mainly by providing access at immobile ions beyond the depletion zones that appear around the roots.

The N concentrations of beech leaves observed in other studies were higher than in this experiment (Balsberg-Pahlsson 1988, Duquesnay et al. 2000, Santa Regina et al. 2001, Fotelli et al. 2002, Fotelli et al. 2005). Still, the amounts of N in the EM root tips were two times higher than those found in NM tips. EM fungal mycelia contain substantial N concentrations, most likely related to high amounts of chitin in the fungal cell wall (He et al. 2007, Koide and Malcolm 2009, Trocha et al. 2010). It is possible that the high N and ^{15}N concentrations in EM root tips are associated with high metabolically process such as ion uptake and transport, or transfer of carbohydrates (Koide and Malcom 2009, Trocha et al. 2010).

Overall, EM plants had lower N concentrations than NM plants. But ^{15}N labeling application revealed that ^{15}N uptake per plant was higher in EM plants than in NM ones. Specific ^{15}N uptake was significantly higher only in EM plants grown under drought stress. Perhaps, the effect of EM presence with respect to increased plant N concentration would have been stronger, if the drought stress had been applied for a longer period. However, Jones et al. (1991) developed a root bioassay test for N status of the forest trees that showed that plant uptake capacity is inversely correlated with its internal N concentration. In plants with high N status, N uptake mechanisms become down regulated. Our findings were consistent with this theory.

Concerning ^{15}N uptake, the results answered our first two questions: EM colonization increased specific ^{15}N uptake in plants grown under drought stress in full light, and had no significant effect on plants grown in shade.

The presence of EM induced a significant delay in the decline of leaf Ψ_{pd} of beech plants grown in shade and exposed at reduced water availability. These plants had also the highest percent of EM root tips, about 39%. Plants grown in shade and exposed to low soil water contents developed slowly drought stress than plants grown in full light (Holmgren 2000, Lemoine et al. 2002, Rodriguez-Calcerrada et al. 2010), but in our experiment this situation was observed only for EM plants. This result suggests that EM colonization may play a role in plant water relations, similar with findings of other authors (Davies et al. 1996, Morte et al. 2001, Hasselquist et al. 2005).

The influence of EM fungi on root architecture by stimulation of new tips formation may improve N uptake. However, the highest number of root tips was found in plants grown under drought in full light and these plants displayed the highest N uptake. The increase, under drought, of root tips number, while NM root tips fraction decreased and other fractions were stable indicate the rapid fungal colonization of new formed tips. Furthermore, we found a positive correlation between ^{15}N amounts in root tips and in above and belowground plant biomass.

EM plants displayed also larger root surface area and root length, compared with NM plants. These results are consistent with other studies (e.g. Rousseau et al. 1994, Hetrick 1991, Ditengou et al. 2000, Boukcim et al. 2001, Pregitzer et al. 2002, Felten et al. 2009). Shading decreased root size (surface, lengths and number of tips) and also root biomass, resulting in a relatively higher investment in aboveground biomass probably as a shade adaptation (Bloom et al. 1985, Van Hess 1997, Van Hess and Clerk 2003, Coll et al. 2004). Gansert and Sprick (1998) also reported in beech a strong reduction of root growth associated with shading.

In our experiment, we found no effects of shade on EM colonization rate. ^{15}N contents in the sum of root tips of plants grown in shade were lower than those of plants grown in full light. Moreover, plants grown in shade revealed likewise the lowest ^{15}N uptake per plant and also per unit biomass. These results were likely caused by a fraction of inactive EM tips under shade, because of insufficient C supply. Although, shade had no effect on the whole plant total N concentration, the leaf N concentration was significantly greater in beech plants grown in shade than in plants grown in full light. Therefore, N partitioning patterns changed in plants grown under shade. Total N concentrations were consistent with the amounts of ^{15}N allocated to leaves of shade plants. In agreement with the optimal partitioning theory, plants should allocate extra biomass to those organs which are involved in the acquisition of resources that most limits growth (Bloom et al. 1985). N is

required for production of light-capturing pigments leading to a positive correlation between photosynthetic rates and foliar N concentrations (Reich et al. 1997). Therefore, under shade, beech increase both leaf mass and foliar N (Kobe et al. 2006, Kobe et al. 2010). ^{15}N partitioning was also significantly influenced by EM presence in the sense of increasing ^{15}N contents in roots in EM plants compared with NM ones. However, ^{15}N amounts in EM root tips were greater than those of NM root tips. Due to the active metabolically processes such as N uptake and assimilation, the N demand of EM roots were probably higher than those of NM ones and the plant was forced in this case to allocate more N belowground (Gessler et al. 1998b).

This study leads support to the view that the positive effects of EM symbiosis on N uptake in beech may be invalidated by long-term reduced C productivity under shade, but not by short-term reduction induced by drought stress. Moreover, EM colonization changed root architecture under drought conditions, in the sense of improving N uptake.

4.5. References

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CHAPTER 5: Girdling affects ectomycorrhizal fungal diversity and reveals functional differences of EMF community composition in a beech forest

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5.0. Abstract

The relationships between plant carbon resources, the soil carbon and nitrogen content, and ectomycorrhizal fungal (EMF) diversity in a mono-specific old-growth beech forest (*Fagus sylvatica*) were investigated by manipulating carbon flux by girdling. We hypothesized that disruption of the carbon supply would not affect diversity and EMF species numbers if EMF can be supplied by plant internal carbohydrate resources or would result in selective disappearance of EMF taxa because of differences in carbon demand of different fungi. Tree carbohydrate status, root demography, EMF colonisation and EMF taxa abundance were measured repeatedly during one year after girdling. Girdling did not affect root colonization but decreased EMF species richness of estimated 79-90 taxa to about 40 taxa. *Cenococcum geophilum*, *Lactarius blennius* and *Tomentella lapida* were dominant colonizing about 70% of the root tips and remained unaffected by girdling. Mainly cryptic EMF species disappeared. Therefore, the Shannon-Wiener index (H´) decreased but Evenness was unaffected. H´ was positively correlated with glucose, fructose and starch concentrations of fine roots and also with dissolved organic carbon-to-dissolved organic nitrogen ratio suggesting that both H´and DOC/DON were governed by changes in belowground carbon allocation. Our results suggest that beech maintains numerous rare EMF species by recent photosynthate. These EM fungi may constitute the insurance for adaptation to changing environmental conditions and maintenance of taxa previously not known to colonize beech may provide an ecosystem service for future forest development.

Key words: carbon flux, *Cenococcum geophilum*, diversity, ectomycorrhiza, insurance hypothesis

5.1. Introduction

In temperate and boreal forest ecosystems, most tree species form ectomycorrhizal fungal (EMF) associations. EM fungi ensheath the root tip forming mantle-like structures with a typical appearance (Agerer 1987-2006). The presence and lengths of hyphae emanating from the mantle are characteristic of different EMF species and establish different soil exploration types (Agerer 2001). It has been assumed that EMF communities are specifically adapted to mobilize sparse soil nutrient resources in boreal and temperate forests (Buscot et al. 2000, Read et al. 2003). Current estimates indicate that about 80% of all nitrogen and phosphorus present in plants have been taken up via mycorrhizas (Hobbie and Hobbie 2006, van der Heijden et al. 2008, Lambers et al. 2009).

Unlike free living soil microbes, EMF have direct access to reduced carbon from their host plants. Already more than 50 years ago, Melin and Nilsson (Meinen et al. 2009) showed that ^{14}C applied to leaves was recovered within one day in EMF suggesting a strong dependence of fungal metabolism on host photosynthesis. Subsequent isotopic studies corroborated tight connections between current photosynthate and EMF (Leakeet al. 2001, Heinosalo et al. 2004). EMF hyphae constitute the main path of plant-derived carbon into the soil (Godbold et al. 2006, Hobbie 2006). Furthermore, EMF hyphae contribute substantially to soil respiration (25% from hyphae and 15% from roots) (Heinemeyer et al. 2008). As hyphal respiration decreased strongly in response to girdling of trees a tight metabolic link between extramatrical mycelia and host photosynthetic activity must exist (Högberg et al. 2001, Bhupinderpal-Singh et al. 2003, Andersen et al. 2005). In addition, fruiting body formation of EMF species was strongly dependent on host photosynthetic capacity (Högberg et al. 2001, Kuikka et al. 2003). In contrast, the significance of current assimilate supply for EMF colonization of root tips and their community composition is not yet well understood. Since trees contain substantial stores of carbohydrates in roots and stem (Barbaroux et al. 2003), it may be expected that EM fungi can be maintained if this carbon resource was available. For example, defoliation experiments with conifers, which restricted but did not eliminate current photosynthate transfer to roots, showed no effects on root EMF colonization; however, under conditions that negatively affected above ground biomass production, morphotypes with thick mantles were suppressed compared to those with thin mantles suggesting a shift to less carbon demanding EMF species (Kuikka et al. 2003, Markkola et al. 2004, Saikkonen et al. 1999, Saravesi et al. 2008) and shift of EMF species abundance between girdled and non-girdled trees (Cullings et al. 2001). Earlier

studies also reported decreased EMF colonization of root tips (Gehring et al. 1997, Rossow et al. 1997).

In a common garden experiment with young beech trees strong shading over several years, that severely limited plant growth, suppressed EMF colonization and resulted in low EMF diversity (Druebert et al. 2009). Since nutrient deficiencies were excluded by growing the trees in nutrient rich compost soil, Druebert et al. (Druebert et al. 2009) concluded that photosynthetic activity was the driver for mycorrhizal colonization. As EMF abundance and diversity were directly correlated in this study, it remained open whether beech carbon productivity was also the driver for diversity. However, EMF community composition was strongly affected by shading and slightly by short-term girdling suggesting that EMF taxa are sensitive to changes in plant internal carbohydrate resources (Druebert et al. 2009). Since the interaction of EM fungi and their hosts also depends on their surrounding environment, the significance of photo-assimilates for EMF abundance, diversity and community composition remains to be shown for adult forest trees which have usually high EMF diversity and low nitrogen availability (Taylor et al. 2000, Buee et al. 2005, Rumberger et al. 2004, Grebenc et al. 2007).

The aim of this work was to test the hypothesis that EMF abundance and diversity are independent of current photo-assimilate supply and can be maintained by internal resources. To investigate this concept old-growth beech trees (*Fagus sylvatica* L.) were girdled to suppress carbon allocation to roots. Since disruption of current assimilate flux affects carbohydrate source strength, we hypothesized that changes in EMF taxa composition would occur if EMF species have different carbon demand. Tree carbohydrate status, root demography, EMF colonization and EMF taxa abundance were measured repeatedly during one year after girdling. Since girdling also affects carbon release into and probably nutrient uptake from soil, the influence of possible feedbacks by changes in the organic carbon to nitrogen ratio of the soil on EMF diversity was also assessed.

5.2. Materials and methods

5.2.1. Site description

The experimental site is located in the Swabian Jura in south-west Germany 800 m a.s.l. (47°59`N, 8°45`E). Mean annual air temperatures, measured at 1.5 m above ground level (humicap HMP45D, Vaisala, Helsinki, Finland) in the study years 2006 and 2007 were 7.7°C and 7.0°C, respectively. The sums of annual precipitation, measured with a tipping bucket (ARG 100, Vaisala, Helsinki, Finland), in 2006 and 2007 were 729 mm and 824 mm, respectively.

Six plots each consisting of 5 adjacent trees were established in an 80- to 90-yr-old beech (*Fagus sylvatica* L.) forest. Soil profiles were characterized as Rendzic Leptosols derived from limestone with high fractions of stones and rocks. pH values were measured in 0.01 mM $CaCl_2$ and ranged between 6.5 and 7.1. For determination of total carbon and total nitrogen concentrations, soil of the Ah horizon was dried, sieved (< 2 mm), and subjected to dry combustion using an elemental analyser (Vario EL, Elementar Analysensysteme GmbH, Hanau, Germany). Inorganic carbon concentrations of samples were determined after ignition of samples for 4 h at 550°C. The concentrations of organic carbon were calculated as the difference between total and inorganic carbon. The Ah horizon is characterized by high organic C content (11.0 ± 0.4%), high N content (0.8 ± 0.1%).

Soil temperatures were measured continuously at 10, 30, 50, 100, and 200 mm depth using PT100 probes. Mean temperature data from these profiles are shown in Table 1. Soil moisture was determined using two probes according to the time domain reflectometry method (TDR, CS615, Campbell Sci., Shepshed, GB). The probes, which have a sensor length of 0.3 m, were buried vertically in the ground, thus covering the uppermost 0.2 m of the slope parallel ground. The TDR soil moisture recordings were compared with gravimetrically taken soil moisture measurements to allow a soil-specific calibration. Soil moisture was indicated as means from both probes (Table 5.1). Further details of the site, stand and soil have been described elsewhere (Holst et al. 2004, Dannenmann et al. 2007).

Table 5.1: Mean soil temperature, soil moisture and precipitation during the week before harvest and at the harvest dates. Soil temperature is a mean of measurements at 1, 3, 5, 10 and 20 cm soil depths.

Harvest day	Temp (°C)[a]		Moisture (%)[b]		Precipitation (mm)	
	Week before	Harvest day	Week before	Harvest day	Week before	Harvest day
10.October 06	10.9	11.4	53.0	50.8	1.06	0.00
14.May 07	10.1	10.2	50.3	47.9	3.40	6.78
15.August 07	13.6	15.0	53.0	48.5	1.87	0.77
09. October 07	11.7	10.4	49.3	47.8	0.00	0.00

[a]Soil temperature is a mean of measurements taken at depths of 1, 3, 5, 10, and 20 cm.
[b]Soil moisture is the mean value from measurements taken at the upper 30 cm.

5.2.2. Girdling and sampling scheme

On 9th August 2006, on three of the six plots five trees grouped as pentagon (see scheme in supplement 5.1) were girdled approximately 1.2 m above ground by complete removal of an approximately 83 mm wide bark strip. The other three plots, each containing five trees grouped in a pentagon, served as controls. At each sampling date, one soil core of a volume of 1004 ml (0.08 m diameter, 0.2 m height, which corresponds to the Ah horizon) was harvested at a distance of about 1 m at each of the selected trees resulting in 15 soil cores per date and treatment. Since about 80% of fine root biomass is present within a distance of 2 m of the stem (Melot 1986), it was assumed that the majority of fine roots belonged to the closest tree. Soil cores were collected in October 2006, May 2007, August 2007 and October 2007 on each plot yielding a total of 30 samples per harvest date. An exception was October 06, where mixed samples were collected from each of the three plots with girdled trees. The soil cores were kept in polyethylene bags at 4 °C until analysis.

5.2.3. Root analysis and ectomycorrhiza morphotyping

The roots were carefully washed, spread out randomly and four to six roots corresponding to a mean weight of 1.71 g fresh mass were randomly removed from the mixture. All root tips of this sample were counted using a stereomicroscope (Stemi SV 11, Zeiss, Jena, Germany). The number of root tips per sample ranged between 700 and 900. Live and dead roots were separated according to Allen et al. (2000). Vital and dead ectomycorrhizal root tips were sorted as described by Downes et al. (1992).

Different types of ectomycorrhizae were morphological classified following the procedure and identification keys of Agerer (1987-2006). Each morphotype was described and photographed (Coolpix 4500, Nikon, Tokyo, Japan). To calculate mantle surface, the photographs of the morphotypes and of a scale bar were used to assess the diameter (d) of the mycorrhizal root tips and length (Agerer 1987-2006) of coverage with the hyphal mantle. Surface was calculated as d x π x l. EM taxa were classified after (Agerer 2001) as contact type (no hyphae), short to intermediate distance exploration type (hyphae) and long distance explorations type (rhizomorphs present) based on the description of the morphotypes (supplement MT). Ten to 20 root tips of each morphotype were collected and kept frozen at -80°C until molecular identification.

5.2.4. ITS-sequencing

About five to ten root tips of each morphotype were used for total genomic DNA isolation using the plantDNA-OLS kit (OLS OMNI life science, Hamburg, Germany) following the manufacturer's

instructions. Before extraction, samples were ground in liquid nitrogen with a ball mill (Type MM2, Retsch, Haan, Germany). Isolated DNA was used as a template for PCR amplification of the internal transcribed spacer (ITS) region of the fungal ribosomal DNA. We used the primers ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') according to White *et al.* (1990). PCR was conducted as described previously (Drueber et al. 2009). PCR products were separated by electrophoresis on a 1.5% agarose gel supplemented with ethidium bromide and visualised under UV light (Fluor-STM Multiimager, BioRad, Munich, Germany) as described by Sambrook & Russell (2001). When no PCR products were found the procedure was repeated if frozen morphotypes still were available. Single band PCR products were purified for DNA sequencing using the straightPCR-OLS kit (OLS OMNI life science, Hamburg, Germany). When the PCR resulted in more than one amplification product, the PCR products with the expected size were cloned into a pGEM-T vector (pGEM-T System I Promega, Madison, USA). Selection was performed by a blue-white assay on LB agar plates, supplemented with ampicilline (100 µg ml^{-1}). The transformants were analysed for the presence of plasmid DNA by ITS region re-amplification. PCR products of expected size were purified by precipitation with isopropanol for 1 h at room temperature, followed by 30 min centrifugation (17900 g, room temperature, centrifuge 5417 R Eppendorf, Hamburg, Germany) and sequenced. Sequencing was performed by the dideoxy chain – termination method using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Foster City, USA) and an automatic sequencer (ABI Prism 3100 gEnetic Analyser, 36 cm capillary, Matrix Pop 6, Applied Biosystems Foster City, USA).

5.2.5. Analysis of sequences

Sequences were assembled using Staden Package 4.10 (http://staden.sourceforge.net). For fungal identification, BLAST searches were carried out against the NCBI (http://www.ncbi.nlm.nih.gov/) and UNITE (http://unite.ut.ee) public sequence databases. Sequences were assigned with matching species names when the BLAST matches found have showed identities higher than 95% and scores higher than 800 bits. If no appropriate match was found the sequence was assigned with a higher level taxonomic name or was called uncultured EMF and numbered. The sequences have been deposited in NCBI GenBank with the GenBank accession numbers FJ403484- FJ403518, and FJ823388- FJ823391, FJ823393 FJ823394, FJ823396, FJ823397. A phylogenetic tree of nucleotide sequences alignments for the ITS regions was computed by MEGA 4.1 software (60). Phylogenies were inferred by the neighbour–joining method and tested by bootstrapping using 1000 replicates.

5.2.6. Carbohydrate analysis

Bark, phloem exudates, wood, coarse and fine roots sampled in Oct 06 and Oct 07 were used for carbohydrate analyses. Wood comprising about 5 annual rings and bark were collected 50 mm above and below the girdle. Control samples were taken from non-girdled trees at 1.2 m above ground. Phloem exudates were obtained by incubation of fresh bark strips in 2 ml 10 mM EDTA solution for 5 hours (Schneider et al.1996, Gessler et al. 2004). The resulting supernatant was stored at -20°C. Other materials were dried at 60°C and ground with a ball mill (Retsch, MM2000). One ml double deionised water was added to 45-55 mg of sample, agitated at 4°C for 1 h, heated at 95°C for 10 min, cooled down to room temperature and centrifuged (10 min, 12000 g). The supernatant was stored for sugar analysis, the remaining pellet for starch measurements.

The concentrations of fructose, glucose and sucrose in phloem exudation solutions and in plant extracts were determined by high performance liquid chromatography (HPLC; Dionex, Idstein, Germany). 200µL aliquots of the sample were diluted with 500µL of double deionized water. From this mixture 100 µL were injected into the HPLC system. Sugars were separated on a CarboPac PA1 separation column (Dionex, 4x250 mm) with 200 mM NaOH as the eluent and a flow rate of 1 ml min^{-1}. All sugars were identified and quantified with external standards and expressed in glucose equivalents per g of tissue.

Starch was determined using a commercial kit for starch analysis (No. 10 207 748 035; Boehringer-Mannheim/Roche R-Biopharm AG, Darmstadt, Germany). Starch in the pellets was solubilised in dimethylsulfoxide and HCl and hydrolysed by amyloglucosidase. The resulting glucose was determined enzymatically in a spectrophotometer at 340 nm (8)

5.2.7. Calculation of diversity indices and statistical analyses

Statistical analysis was performed using Statgraphics Plus 3.0 (StatPoint, Inc., St Louis, MO, USA). Experimental factors were treatment (girdled/control) and time point (sampling date). The following diversity indices were calculated: species richness H(max) = ln (number of all species), Shannon-Wiener index, $H' = -\sum p_i \ln p_i$, where p is the probability of the species i, and Evenness = H′/ H(max) (Shannon & Weaver, 1949). The sampling unit "soil core" served as the basis for calculating these indices.

The distribution of fungi species in samples was tested by a χ^2 test. The probability of finding a species on y root tips was calculated as $p = 1 - (1-x)^y$, where x is the abundance of the species in community (Taylor 2002). Rarefaction was necessary since roots of control trees contained higher numbers of mycorrhizal root tips than roots of girdled trees. Individual-based rarefied species

richness and H´ were calculated using EcoSim software version 7.72 (Gotelli et al. 2001). Pearson product moment correlations were calculated between each pair of variables as explained in tables or text. Where means are shown, normal distribution of the data was tested by calculation of standardized skewness and standardized kurtosis. Since values of these parameters ranged between -2 to +2, analysis of variance (ANOVA) was followed by a multiple range test. Means were considered to be significantly different from each other, when $P \leq 0.05$. Significant differences are indicated in tables and figures by different letters. The effect of girdling and time on root demography and diversity indices was analysed by repeated measures analysis of variance by the GLM procedure of Statgraphics Plus 3.0 software using trees as subject (StatPoint, Inc., St Louis, MO, USA). Regression analysis were plotted with the programme Origin 7G (Origin Lab, Corporation, Northhampton, ML, USA). Detrended correspondence analysis (DCA) was used to visualize the similarity of EM fungal community between treatments and sampling time points. The analysis was performed with R software version 2.9.2 (R Project for Statistical Computing) with the package "Vegan", using relative abundance of fungal species per time point and treatment as input matrix. For analysis β diversity of EM fungal community composition the Sørensen index was calculated by EstimateS software version 8.2.0 (Colwell 2005).

5.2.3. Results

5.3.1. Girdling affects root demography but not EMF colonization of vital roots

Root tips of mature, healthy beech trees investigated during different seasons displayed the following four fractions: 55 ± 4 % vital mycorrhizal tips, 1.6 ± 1.1 % vital non mycorrhizal tips, 31 ± 1 % dry mycorrhizal tips and 15 ± 3 % dry non-mycorrhizal root tips. These means showed some seasonal fluctuations (Figure 5.1), which were apparently not related to the acute weather and soil conditions (Table 5.1).

Repeated measures ANOVA revealed significant demographic changes of root tips of girdled compared with those of non-girdled control trees (Figure 5.1). The fraction of dead mycorrhizal tips was about 20% higher at roots of girdled than at that of control trees; the fraction of vital EMF was correspondingly decreased (Figure 5.1A, C). The shift towards increased fractions of dead EMF was observed about six weeks after girdling and did not increase further in the following year. In contrast to dead EMF root tips, whose fraction was relatively stable, a time-dependent approximately three-fold decrease in dead non-mycorrhizal root tips was observed (Figure 5.1D, significant interaction of girdling x time). This result suggests differences in the longevity of mycorrhizal and non-

mycorrhizal fine roots and may indicate that the formation of fine roots became slower than their degradation.

When only vital root tips were considered, EMF colonization of roots of non-girdled trees was 97 ± 1% across all sampling dates (n = 28060). Girdling had no significant influence on the fraction of vital EM per total vital root tips (97 ± 2%, P = 0.939), however less vital root tips were present (n = 13435). The fraction of non-mycorrhizal vital root tips was always very small (Figure 5.1B).

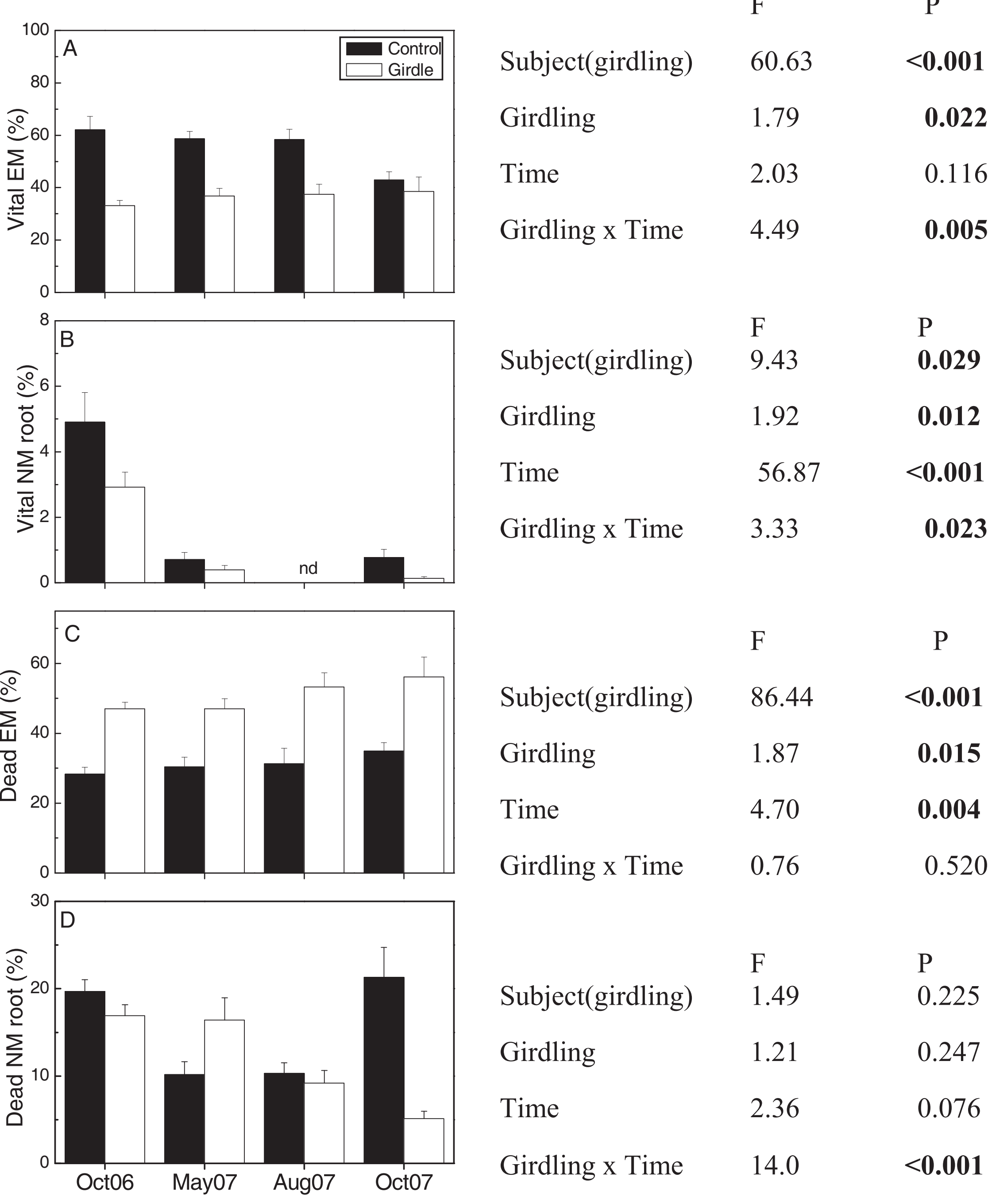

Figure 5.1: Seasonal fluctuations in the demography of root tips of healthy beech trees (closed bars) and after girdling (open bars). Bars indicate means of n = 15 (± SE) per treatment and time point for vital ectomycorrhizal root tips (A), vital non-mycorrhizal root tips (B), dead ectomycorrhizal root tips (C) and dead non-mycorrhizal root tips (D). *F*- and *P*-values obtained by repeated measures analyses of variance are shown next to the figures. *P*-values < 0.05 are shown by bold letters.

5.3.2. Carbon storage pools are depleted slowly after girdling

To investigate whether the persistence of ectomycorrhiza and high colonization rates for more than one year after girdling were related to the consumption of plant-internal carbon storage pools, we determined starch and soluble sugars in below and above-ground tissues (Figure 5.2, Table 5.2). Starch concentrations decreased in coarse and fine roots of girdled trees compared with controls (Figure 5.2A). After about one year still 25% of the maximum starch concentrations found in control roots were present in roots of girdled trees. The concentrations of soluble carbohydrates (glucose, fructose and sucrose) in coarse roots did not decrease significantly (Figure 5.2B). In contrast, in fine roots of girdled trees reductions in soluble carbohydrates occurred at both sampling dates (Figure 5.2B). None of the carbohydrate pools became completely depleted.

The carbohydrate concentrations were also lower in phloem exudates of girdled compared with control trees (Table 5.2). The decrease was stronger below than above the girdle supporting that basipetal transport of carbon was disturbed. Since the stem above and below the girdling site still contained considerable concentrations of soluble sugars and starch, continued transport to roots in the stem at low rates cannot be excluded. However, the transfer rate is very low (Druebert et al. 2009).

Table 5.2: Carbohydrates in phloem and stem of beech (*Fagus sylvatica* L.) of control (C) and girdled (G) trees in autumn 2006 and 2007.

Carbohydrates	C-06	G-06 (a)	G-06 (b)	C-07	G-07 (a)	G-07 (b)
SC in phloem exudate (µmol g^{-1} f.wt.)[§]	26.88 ±1.21d	22.07 ±1.17c	12.12 ± 0.66a	35.84 ±1.39e	26.77 ± 1.26d	15.53 ±0.91b
SC in stem (µmol g^{-1} d.wt.)	93.13 ± 5.44c	96.81 ± 4.69c	71.26 ± 4.25b	119.35 ± 11.43d	44.89 ± 7.61 a	25.99 ± 4.11a
Starch in stem (µmol g^{-1} d.wt.)	49.08 ± 4.94bc	46.43 ± 4.54b	23.13 ± 3.77a	59.70 ± 5.29c	116.53 ± 15.76d	14.15 ± 2.93a

[§] Soluble carbohydrates (SC) are the sum of glucose, fructose and sucrose. In girdled trees the carbohydrate concentrations were determined above (a) and below (b) the girdle. Data are means of n = 15 (± SE). Different letters in rows indicate significant differences at $P \leq 0.05$. All data have been expressed in glucose units.

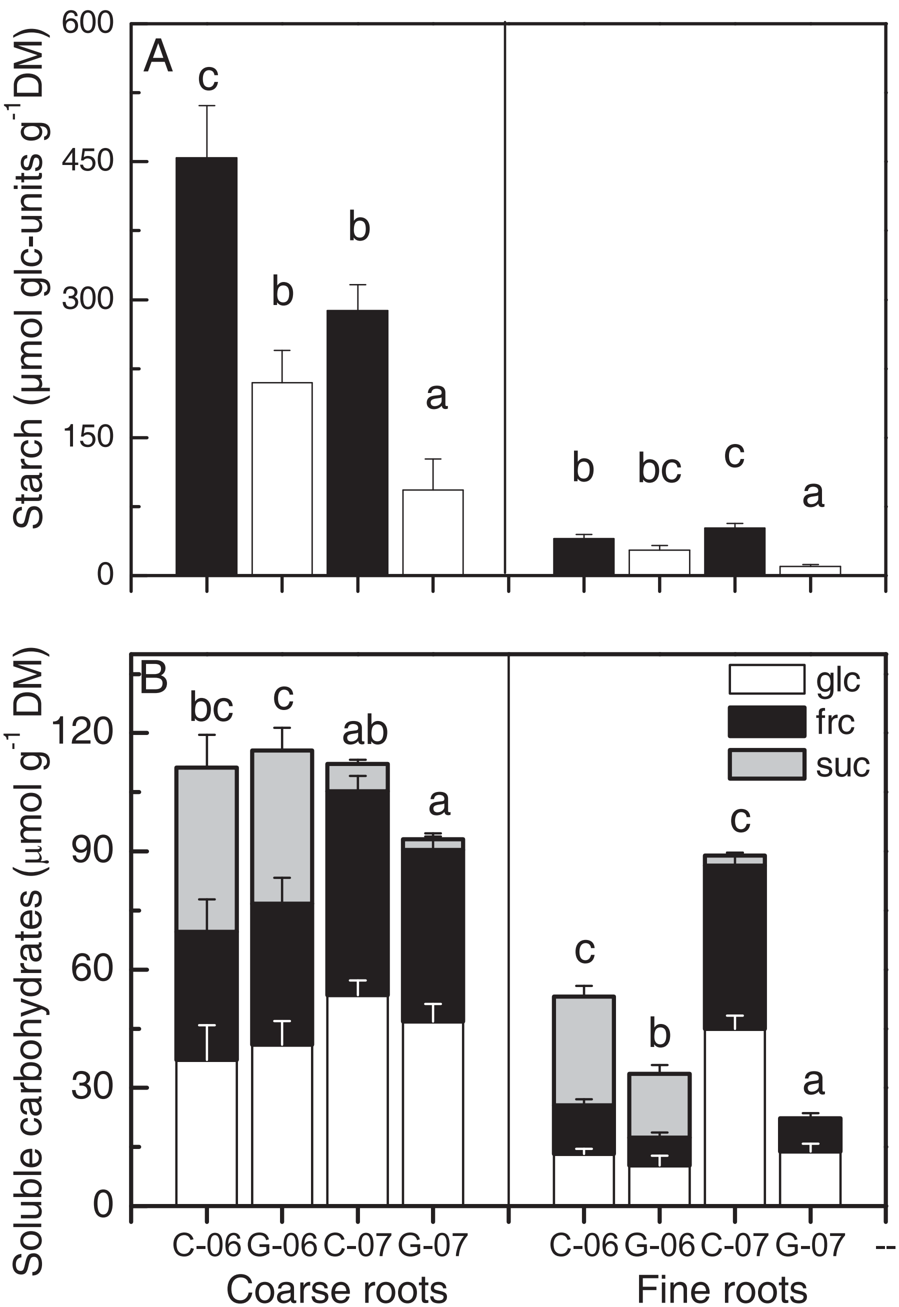

Figure 5.3: Relative abundance of ectomycorrhizal species sorted according to descending rank at roots of girdled (open symbols) and abundance at roots of control beech trees (closed symbols). The relative abundance was calculated for all sampling dates together. Y = number of mycorrhizal root tips. Arrow indicates species not found after girdling (= lost species). Dashed lines indicate the range of lost species with a detection probability of P ≤ 0.99. Details are given in the text.

5.3.3. Girdling leads to loss in ectomycorrhizal species

To investigate whether girdling affected EMF community structures and diversity, we conducted morphotyping and ITS sequencing (Genbank accession numbers, see Table 5.3; morphotypes, see supplement MT). Morphotypes, for which ITS sequence information was not available because PCR products were not obtained, were called MTs. ITS sequence information was obtained for 55 morphotypes that corresponded to 41 different taxa indicating that morphotyping overestimated species numbers by a factor of 1.3 (Table 5.3). ITS sequences not belonging to known taxa were called uncultured ectomycorrhizal fungus (UEM) and numbered consecutively (Table 5.3). We constructed a phylogenetic tree to assign clade names to UEMs and to ascertain the annotation of species names obtained by BLAST (Supplement 5.2). The relative abundance of the sequenced taxa summed up to 90% root colonization for both control and girdled trees.

Assuming that each of the MTs represented one new species, we found a maximum of 89 EMF species at roots of control and 39 at roots of girdled trees. Rarefied species accumulation curves confirmed this massive species loss (Supplement 5.3). Using 1.3 as the correction factor for species overestimation for non-sequenced morphotypes resulted in estimated numbers of 79 and 36 EMF taxa at roots of control and girdled trees, respectively. Since we considered these errors, which were in the range of 11% to 16%, as relatively low, all unidentified MTs were considered as different species for the following calculations and assessments.

The EMF species were sorted according to descending rank order for girdled trees (Figure 5.3). Girdled trees hosted one new species (*Cortinarius* sp. which had highest similarity with *C. solis-occasus,* rank 91 in Figure 5.3). The 50 species that had disappeared from roots of girdled trees contributed together 7.8% to root colonization of control trees. We wondered whether the lost species, most of which occurred only with very low relative abundance at roots of control trees, had a reasonable chance to be observed since roots of girdled trees contained less vital roots tips than those of non-girdled trees (Figure 5.1, see also number for root tips (y) in Figure 5.3). We estimated the theoretical probability of detection of each lost species according to Taylor (2002). As a prerequisite for this analysis, dispersion indices were calculated that showed that the species were not clustered (Supplement 5.4a). Among the 50 species that had disappeared, only nine species (rank 80 to 89) had a detection probability of less than 99%. Furthermore, rare species also showed random dispersion (Supplement 5.4b). This indicates that for the majority of the undetected species disappearance was not a sampling effect.

It should be noted that our experimental design does not completely exclude introgression of roots from distant non-girdled trees. However, the observed species loss together with the decrease in

soluble carbohydrates (Figure 5.2), reduced number of vital root tips, decreases in vital EM and increase in dead EM (Figure 5.1) support that majority of roots were from girdled trees.

In addition to species loss, pronounced changes in the relative abundance of several EMF species occurred at roots of girdled trees compared with controls (Figure 5.3). We considered all species that were outside the boundaries of a 99% confidence interval after linear regression of abundance of EMF at roots of controls versus species abundance of EMF at roots of girdled trees as increased or decreased (Supplement 5.5). Diminished abundances at roots of girdled trees were found for nine putative species (*Tomentella pilosa,*UEM (Russulaceae), UEM (Pezizales), UEM 11 (basidiomycetes), UEM 9 (ascomycetes), MTs 48, 62, 89, and 126 with the rank numbers 28, 29, 30, 31, 33, 34, 35, 36 and 38 in Figure 5.3).

Table 5.3: Ectomycorrhizal taxa identified by ITS sequencing. Data of the best alignment by UNITE (accession number starting with UDB) or NCBI (other accession numbers) and their similarity with BLAST are shown. A indicates abundance classes: a = abundant (>1%) on roots of non-girdled trees, p = present (<1% and >0%) at roots of girdled and non-girdled trees, l = lost at root of girdled trees, n = new at root of girdled trees. Rank refers to rank abundance in figure 3.

Taxon	Number of MT	Length of ITS	Accession number	Similarity with accession	%	Beech* EM	A	Rank
Cenococcum geophilum	2	516	FJ403485	UDB002301	98	yes	a	1
Lactarius blennius	5	521	FJ403484	UDB000363	na	yes	a	2
Tomentella lapida	2	492	FJ403486	UDB001659	99	uc	a	3
Hysterangium nephriticum	3	502	FJ403487	EU784366	100	no	a	4
UEM 2	1	529	FJ403488	UDB001120	96	-	a	5
Hebeloma crustuliniforme	1	537	FJ823391	UDB000931	95	no	a	6
Tomentella subclavigera	3	502	FJ403493	UDB000259	98	uc	a	7
Tomentella lilacinogrisea	1	516	FJ403490	UDB000272	98	uc	a	8
UEM Sebacinacea	1	553	FJ403491	EF218816	95	-	a	9
Tuber puberulum	1	499	FJ403492	UDB000122	99	no	a	10

UEM 4	1	558	FJ403495	EU816686	99	-	a	11
UEM 12	1	537	FJ823397	EF434095	98	-	a	12
Tomentella viridula	1	469	FJ403494	UDB000261	99	uc	a	13
Russula mairei	1	468	FJ823393	UDB000346	99	yes	p	14
UEM 5	1	545	FJ403497	FJ210754	98	-	p	15
UEM 10	1	509	FJ823390	EU645629	99	-	p	16
UEM (Tricholoma)	1	466	FJ823394	UDB001688	99	-	p	17
UEM 8	1	502	FJ403504	EU816663	95	-	p	18
Inocybe hirtella	1	556	FJ403496	AM882932	99	uc	p	19
Melanogaster macrosporus	1	560	FJ403505	UDB001487	92	no	p	20
Xerocomus pruinatus	2	537	FJ403501	UDB000477	97	no	p	21
UEM (Genea)	1	596	FJ823388	EU668290	99	yes	p	25
UEM 6	1	515	FJ403498	AF509964	96	-	p	26
Inocybe pseudoreducta	2	554	FJ403499	EF644109	95	nc	p	27
Tomentella pilosa	1	508	FJ403506	UDB000241	98	no	p	29
Pesizales spp	1	518	FJ403514	AJ969617	99	uc	p	30
UEM 11	1	544	FJ823396	AY112929	96	-	p	31
UEM 9	1	491	FJ403513	EU816668	99	-	p	33
UEM Russulacea	1	441	FJ403508	AJ937998	99	nc	p	35
UEM 7	1	493	FJ403503	EU816655	97	-	l	39
Russula cuprea	1	516	FJ403500	UDB002420	98	nc	l	40
Humaria hemisphaerica	1	525	FJ403510	UDB000988	99	no	l	44
Sebacina incrustans	1	515	FJ403518	UDB000118	99	no	l	45
Lactarius pallidus	1	576	FJ823389	UDB000306	99	yes	l	50
Inocybe glabripes	1	539	FJ403512	UDB000099	90	uc	l	56
Cortinarius scotoides	1	537	FJ403511	UDB000167	96	uc	l	59

Hygrophorus discoxanthus	1	497	FJ403515	UDB000554	98	uc	l	63
Tricholoma lascivum	1	505	FJ403516	UDB000005	99	-	l	67
Cortinarius boulderensis	1	555	FJ403502	UDB001452	95	uc	l	78
Russula foetans	1	493	FJ403509	UDB002424	95	yes	I	88
Cortinarius solis-occasus	1	576	FJ403507	UDB000712	na	uc	n	90

* = after De Roman et al. 2005, uc = unclear, genus was found on beech, but species level was not reported

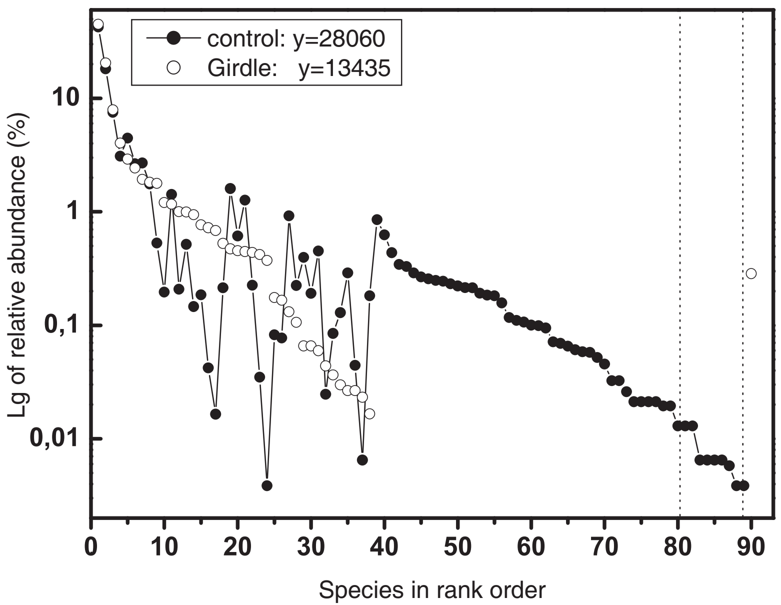

Figure 5.3: Relative abundance of ectomycorrhizal species sorted according to descending rank at roots of girdled (open symbols) and abundance at roots of control beech trees (closed symbols). The relative abundance was calculated for all sampling dates together. Y = number of mycorrhizal root tips. Arrow indicates species not found after girdling (= lost species). Dashed lines indicate the range of lost species with a detection probability of $P \leq 0.99$. Details are given in the text.

Ten putative species occurred with higher abundances at roots of girdled than at those of control trees (*Hysterangium nephriticum*, UEM (Sebacinaceae), *Tuber puberulum*, UEM 5 (Sebacinaceae), UEM 12 (Ascomycetes), *Russula mairei*, UEM 10 (Sebacinaceae), UEM (Tricholoma), MT 44, and MT 67 corresponding to ranks 4, 9, 10, 12, 14, 15, 16, 17, 23, and 24 in Figure 5.3). To find out

whether disappearance or increased abundances were related to mantle thickness of the morphotypes, we estimated the surface of EM species and related this to change in abundance. However, no clear pattern was observed (Supplement 5.6). We also categorized EM according to contact type (no hyphae), short to medium exploration type (hyphae present) and long distance exploration type (rhizomorphs present). Each category lost species, but there was no shift between the categories (Supplement 5.6).

To find out whether species were co-occurring or might have excluded each other, Pearson product momentum correlations were calculated (supplement 5.7). Since there was a large fluctuation of species between sampling dates (see further down), we included only species that were present in at least half of the samples. We found negative correlations between *Cenococcum geophilum* and *Tomentella lilacinogrisea* and an unknown morphotype (UEM2, Phalales). UEM2 was positively correlated with *Tomentella lilacinogrisea* and with *Tomentella pilosa* (Supplement 5.7). *Lactarius blennius* showed a negative correlation with UEM2. Negative correlations were also found for *Tomentella lapida* and *Russula mairei* as well as for UEM (Hebeloma) and *Tomentella subclavigera* (Supplement 5.7).

5.3.4 Girdling affects seasonal fluctuations in diversity and species richness but not in Evenness.

We analysed the influence of time after girdling on the Shannon-Wiener index (H´) and on species richness (S) to find out whether species loss was gradually progressing. Since girdling resulted in lower numbers of vital root tips (Figure 5.1), H´ and S were analysed on rarefied data showing that both indices were generally lower for girdled than for control trees (Figure 5.4A, B). A pronounced decline with progressing time was not found. Instead, H´ and S of controls showed significant fluctuations suggesting that the formation of a more diverse EM flora was favoured by intact trees in autumn compared with other seasons. These fluctuations were not related to mean soil humidity, which was relatively stable at the different sampling dates (Table 5.1).

Evenness, a measure for the dominance structures of species, remained unaffected by girdling (Figure 5.4C). The relative stability of this parameter was caused by a group of seven major EMF species (Figure 5.3), which contributed together 80% of mycorrhizal root tips of control and girdled trees. Among these species *Cenococcum geophilum* was by far the most dominant fungus with a relative abundance of about 40% (Figure 5.3). Six additional species contributed together further 40% of colonization (*Tomentella lapida*, *Lactarius blennius, Hysterangium nephriticum*, UEM2 (Phales), UEM (*Hebeloma*) and *Tomentella lilacinogrisea*).

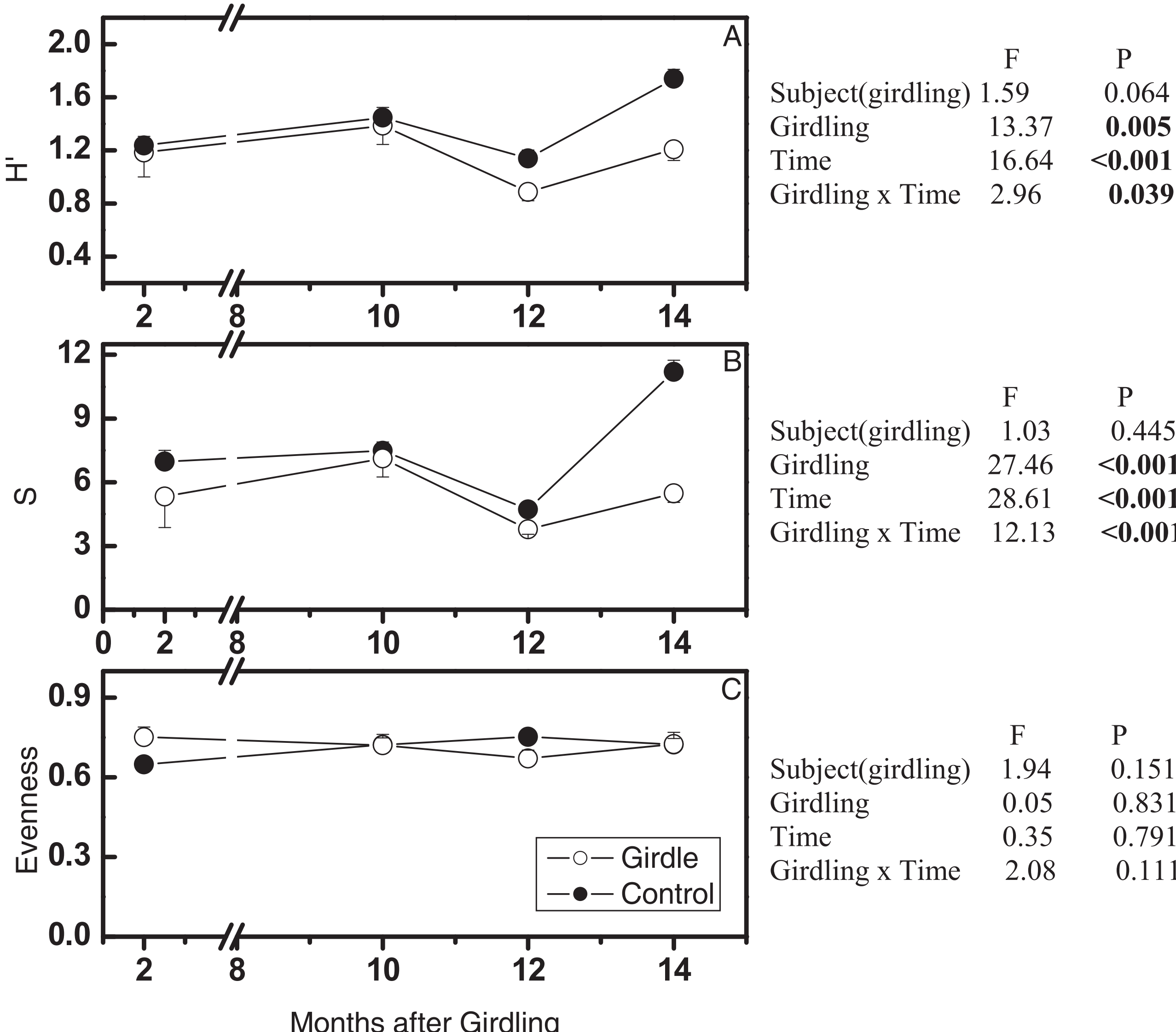

Figure 5.4: Seasonal fluctuations of rarefied Shannon Wiener index H´ (A), species richness S (B) and Evenness (C) of ectomycorrhizal fungal species on roots of girdled (open symbols) and control (closed symbols) beech trees. n = 15 ± SE, except girdled trees at 2 and 10 months after girdling where n = 3 and 7, respectively. For each sample 100 ± 10 individuals were registered. *F*- and *P*-values obtained by repeated measures analyses of variance are shown next to the figures. *P*-values < 0.05 are shown by bold letters.

Detrended correspondence analysis showed that EMF communities were clearly separated according to seasonal fluctuations and by girdling along the first and second axis (DCA1, and DCA2) with eigenvalues of 0.2465 and 0.196, which explained 39.6% and 31.6% of the proportion of variation,

respectively (Figure 5.5). The analysis indicates that the influence of seasonal fluctuations (DCA2) on community composition was larger than that of girdling (Figure 5.5). Analysis of β diversity, measured as Sørensen index, confirmed that the similarity of EMF communities between girdled and healthy trees was greater (0.52 to 0.78) than between sampling dates (0.26 to 0.50, Supplement 5.8).

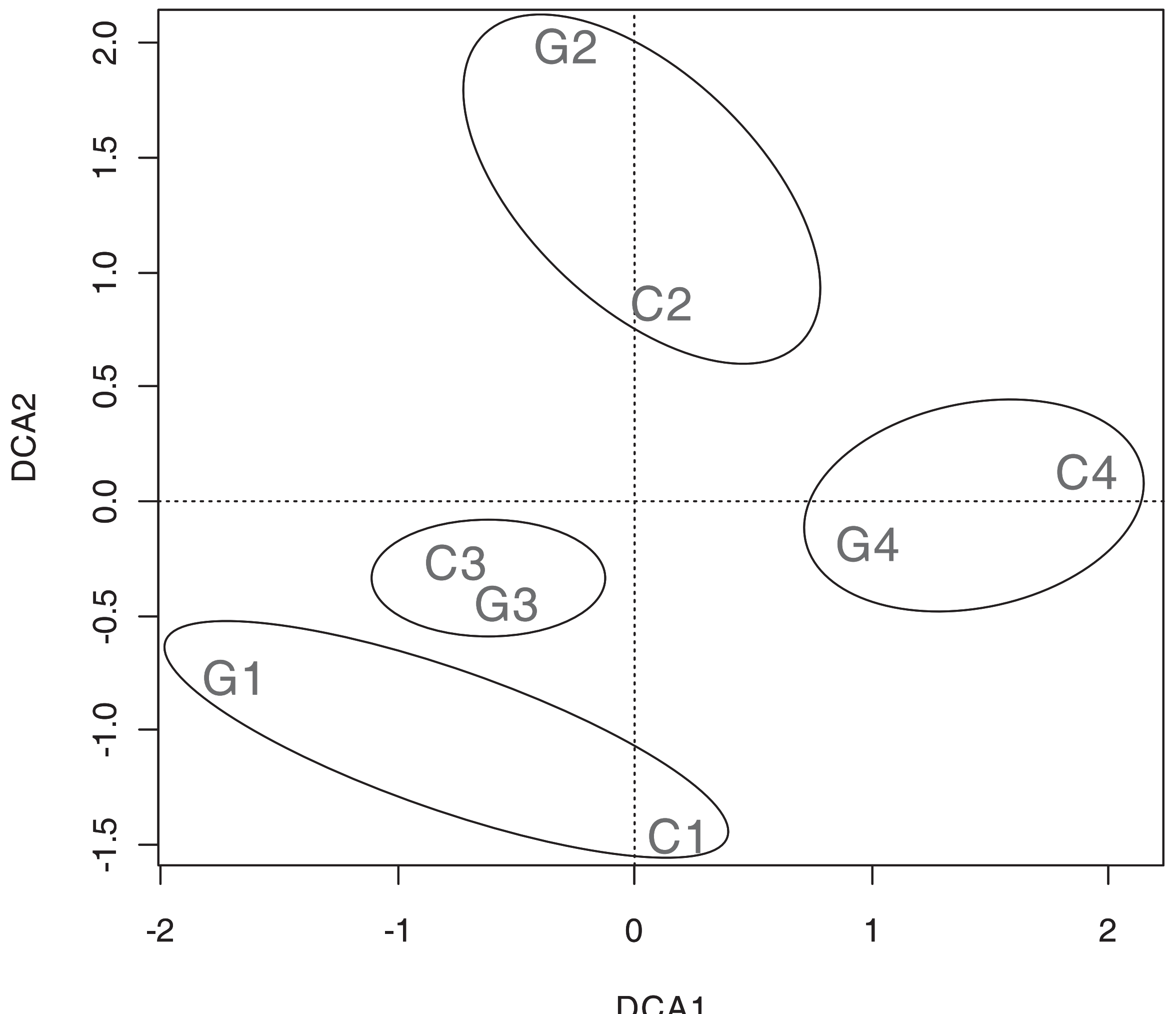

Figure 5: Detrended correspondence analysis (DCA) of EM fungal communities on roots of healthy (C) and girdled (G) trees. Numbers represent the sampling time points (1 = October 06, 2 = May 07, 3 = August 07, 4 = October 07).

5.3.5. Diversity is related to root carbohydrate status and the ratio of dissolved organic carbon-to-dissolved organic nitrogen in soil

We investigated whether the decrease in H´ was correlated with carbohydrate concentrations in fine roots. Regression analysis showed a positive significant relationship between H´ and the concentrations of starch ($P = 0.038$), fructose ($P = 0.003$), glucose ($P = 0.001$) and the sum of the latter two carbohydrates in fine roots (Figure 5.6A). No correlation was found between sucrose levels

and H´ (P = 0.156). The same was true for H_{max} (not shown). Evenness showed no significant regression with any of the carbohydrates (not shown).

We also tested whether individual EMF species were significantly correlated with root carbohydrates. For this analysis, only data sets were used which contained a given species in at least half of the samples. *Cenococcum geophilum* decreased with increasing glucose concentrations (P = 0.050), whereas *Tomentella pilosa* (P = 0.009) and a species of the Russulaceae (P =0.012) whose overall abundances were, however, low (range 0 to 1.6%) increased. The later two species also showed significant positive regression curves with fructose (P = 0.002, P = 0.016).

The starch concentration was significantly correlated with glucose (P = 0.027) and fructose concentrations (P = 0.007) in fine roots suggesting a tight connection between these pools. Soil nitrogen was relatively stable (total N: 7.7 ± 0.7 and 8.6 ± 0.6 mg g^{-1} soil d.wt. on girdled and control plots, dissolved organic N: 29 ± 6 and 32 ± 6 µg g^{-1} soil d.wt. on girdled and control plots, respectively), whereas the dissolved organic carbon was lower in soil from the girdled than in that from non-girdled plots (16). H´ was positively correlated with DOC/DON (Figure 5.6B) but not with soil organic carbon (P = 0.178) or soil nitrogen (P = 0.638).

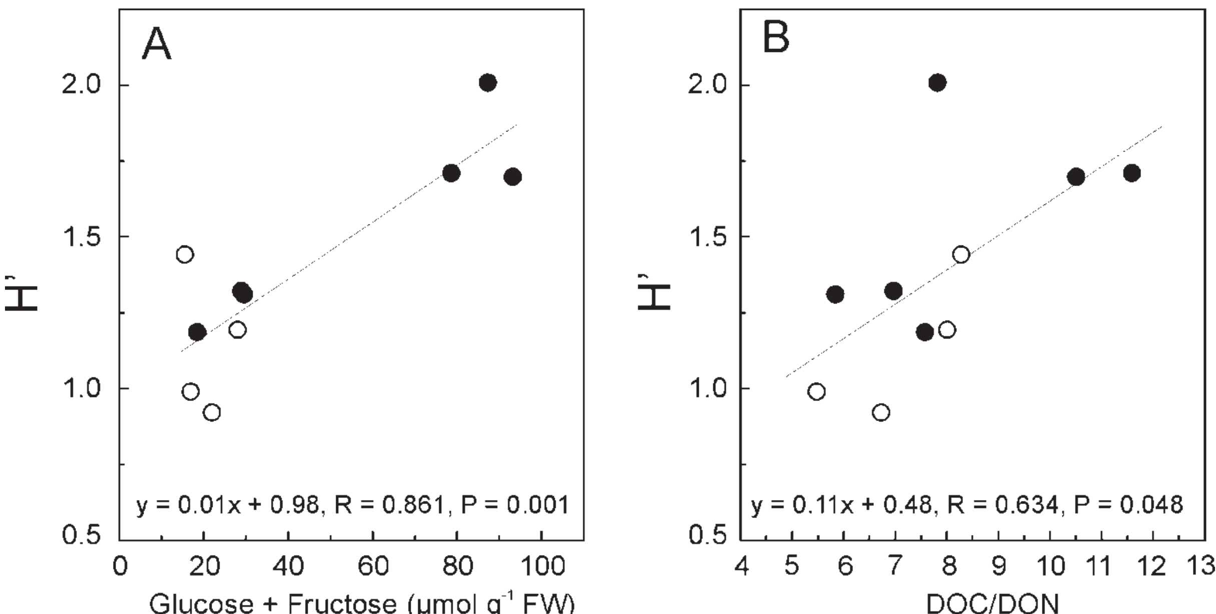

Figure 5.6: Regression between the Shannon-Wiener index H´ and the sum of glucose and fructose (A), and the ratio of dissolved organic carbon to dissolved organic nitrogen in the soil (DOC/DON) (B). Data for DOC and DON were taken from (16). H´ is given as the mean of five soil cores collected per plot in October 06 and October 07, respectively. An exception was October 06 where only one pooled data set across all three plots was available for girdled trees. Closed symbols: controls, open symbols: girdled trees.

5.4. Discussion

5.4.1. Recent assimilates as driver of EMF diversity

The main questions were how root colonization, EMF species composition and diversity were affected when assimilate flux to the roots was suppressed by girdling. On the healthy trees of this study EMF species richness was similarly high as in other stands of adult European beech (Taylor et al. 2000, Buee et al. 2005, Rumberger et al. 2004, Grebenc et al. 2007, Courty et al. 2008) and showed a typical distribution with few abundant and many rare species (Allenn et al. 2009). EMF species composition fluctuated between sampling dates (Figure 5.5), probably because of changing environmental conditions such as temperature and soil humidity (Swaty et al. 1998, Buee et al. 2005, Courty et al. 2008). After girdling root colonization remained unaffected as expected because the internal carbon resources decreased but were not depleted during the time course of this study. This is an important finding because the rapid decreases in extramatrical hyphal respiration, which were observed under pine and beech after girdling (Högberg et al. 2001, Andersen et al. 2009), might have indicated that EMF were cut off the carbon supply chain.

However, despite the presence of significant carbohydrate pools in the root system and support of EMF abundance, which suggests that there was still carbon supply, EMF diversity was strongly diminished. This is in contrast to previous results where girdling of young beech trees did neither affect EMF abundance nor diversity (Druebert et al. 2009). However, in the previous study the trees were grown compost soil, where the typical EM flora of forest trees did not develop and where EMF species richness was three to four times lower than in the present investigation. Johnson et al. (Johnsonet al. 2005) discussed that plants may regulate diversity and community structure of mycorrhizal fungi. Our study suggests that this may occur via carbon supply because diversity and species richness were correlated with glucose and fructose concentrations and with starch in fine roots. The latter observation was surprising at the first glance but may simply reflect the fact that soluble pools of fructose and glucose were directly fed by starch degradation. After girdling, the decrease in sucrose, which is the major transport form for carbon, was most pronounced, however, not correlated with diversity. This appears reasonable because EMF can use monosaccharides such as glucose or fructose but not the disaccharide sucrose as carbon source for their nutrition (Nehls and Hampp 2000).

Notably, girdling affected mainly subordinate taxa. It is possible that they disappeared as a direct consequence of their missing food source or that they were outcompeted by the dominant EMF species. The abundance of the most frequent fungus, *C. geophilum* was inversely correlated with

plant carbohydrates, which points to low carbon demand and high competitiveness of this species. Since *C. geophilum* can also produce mycotoxins (Baar et al. 2000) it may shape EMF communities by affecting its competitors. Indeed, negative relations between *C. geophilum* and other EMF taxa have been observed (Koide et al. 2005). In our study *C. geophilium* was negatively correlated with UEM2 and *Tomentella lilacinogrisea.* However, we cannot distinguish whether *C. geophilum* suppressed these species or whether these species were reduced because the assimilate flow was abolished. The latter option cannot be excluded because the abundance of *T. lilacinogrisea* was linked with root sugar concentrations.

Regardless the underlying mechanisms, our data demonstrate the importance of recent photo-assimilates for EMF species richness. This contention is also supported by the observation that tree seedlings close to undisturbed trees maintained higher EMF species richness and diversity than those grown close to dead stumps or wood debris (Dickie et al. 2002, Iwanski and Rudawska 2007). The community of fungi close to stumps or wood debris was shifted towards EMF with saprotrophic features (Iwanski and Rudawska 2007) in support of the idea of a biotrophy-saprotrophy continuum of EMF fungi (Koide et al. 2008). However, we have no evidence for such shifts, probably because carbohydrate reserves had not yet been used up. After clear-cutting significant effects on EMF diversity of roots of vital stumps were also observed (Jones et al. 2003). However, this treatment affects microclimatic conditions and, therefore, the significance of recent photo-assimilates for EMF diversity was unclear. Changes in plant carbon production may affect soil properties (Gotelli et al. 2001, Högberg et al. 2007) and thus affect EMF diversity by feed-backs effects through changes in soil composition. High soil fertility caused by high nitrogen decreases mycorrhizal diversity (Lilleskov et al. 2002, Parrent et al. 2006). In our study an influence of nitrogen was excluded because total soil N content and dissolved organic nitrogen did not change in response to girdling, whereas DOC decreased after girdling (Dannenmann et al. 2009). The decrease in DOC was probably related to decreased root exudation because a substantial part of dissolved OC was found to originate from photo-assimilates (Giesler et al. 2007). Therefore, the correlation between H´ and DOC/DON is unlikely to reflect a direct influence of properties of the soil solution on EMF diversity but suggests that both DOC and EMF species composition were governed by carbon allocation to roots.

5.4.2. Functional ecological significance of diversity

An important result of our study was that maintenance of EMF species was selective. As the three most abundant species *C. geophilum*, *L. blennius* and *T. lapida* were unaffected by girdling despite

considerable differences in mantle surfaces (No. 1, 2, and 3 in Supplement 5.6), our analysis does not support that voluminous EMF structures *per se* were more sensitive to reduced carbon flow than those with smaller surfaces. EM taxa with rhizomorphs classified as long distance exploration types as well as contact types with smooth mantles disappeared more frequently than those with hyphae (Supplement 5.6). In general, there is no correlation between the amount of extramatrical mycelium of an EM-forming fungal species and its abundance on root tips (Kojller et al. 2006). Preferential allocation of carbon to more efficient extramatrical mycelia has been shown (Rosling et al. 2004). While better service to the plant as afforded by higher outreach for nutrient foraging may be associated with fitness cost and lead to selective species loss, it must be noted that the reduced carbon flow in our study did not cause shifts between the different EM categories since only rare species disappeared. The independence of the functional categories from actual carbon flow suggests that the EM community is adapted to maintain nutrient flow under the prevailing ecological conditions.

The disappearance of *ca*. 50 fungal taxa, which colonized only about 8% of the root tips, suggested that they thrive on excess of recent photosynthate. These fungi may be a legacy for adaptation of the forest to changing environmental conditions. This would support the insurance hypothesis of diversity because rare species may become important for maintaining nutrient supply of beech when microclimatic or soil conditions change. Perhaps rare or undetected species at roots of healthy trees such as *Cortinarius* sp. (similar to *C. solis-occasus*), and *Tuber puberulum* that became more abundant at roots of girdled trees fulfil such functions. The host range of these species must be wider than previously thought because they have so far only been described as EMs of conifers (Melin and Nilsson 1957, De Roman et al. 2005).

The observed instability of many individual EMF species-host interactions in our study may indicate that beech is not their preferential host. One possible ecosystem benefit would be to facilitate the establishment of other tree species in future succession stages of the forest. This idea is supported by the fact that some of the rare taxa have previously been reported to form EMF with conifers (*Humaria hemisphaerica, Hygrophorus discoxanthus*) or with other angiosperms like *Carpinus betulus* or *Tilia* spec. (*Sebacina incrustans*) but not with beech (De Roman et al. 2002). In conclusion, we suggest that a surplus carbon "donated" to non-host EMF taxa would prevent extinction of these communities and constitute an important ecosystem service of mature beech trees.

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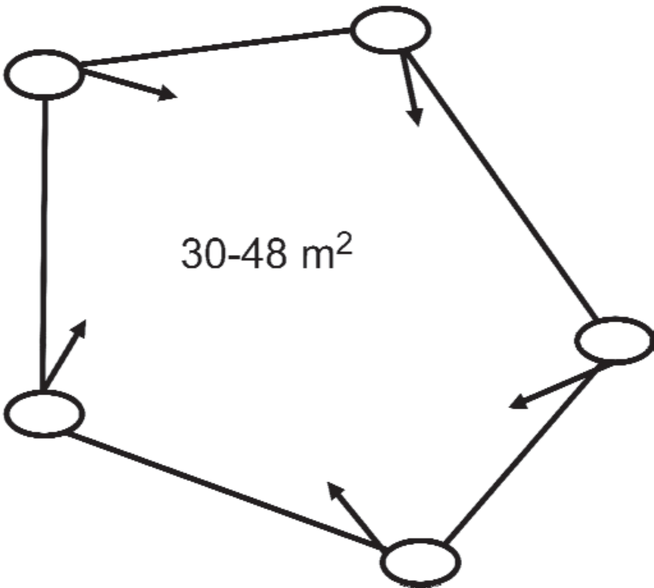
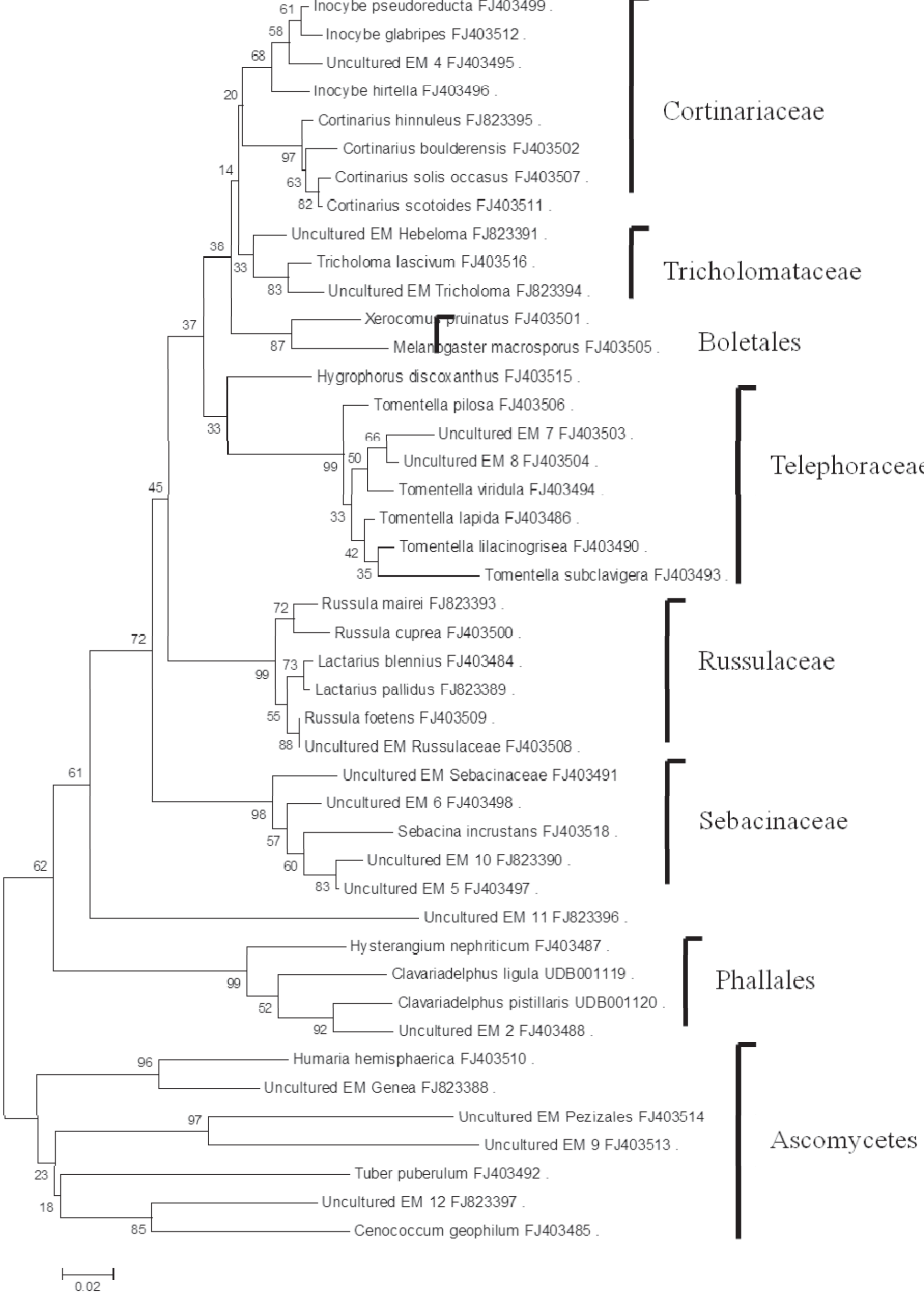
Supplement 5.1

Schematic example of one experimental plot, consisting of 5 adjacent trees. Arrows show the direction of sampling points.



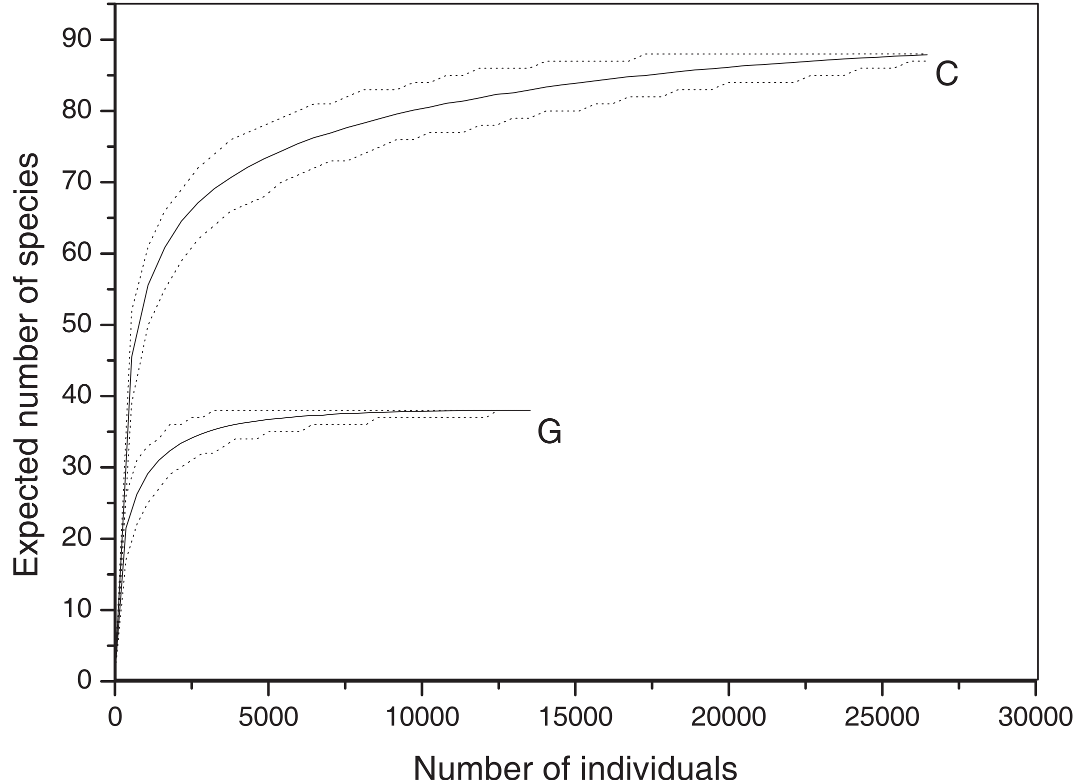
Supplement 5.2

Neighbor-joining tree representing the phylogenetic relationships of EM fungal ITS sequences from control and girdled trees. Accession numbers of GeneBank nucleotide database are given for all sequences. Two sequences of Clavariadelphus (accession number started with UDB) were obtained from UNITE data base and included in phylogenetic tree to permit construction of a Phalalles order and assignment of two Uncultured EM sequences.



Supplement 5.3

Individual-based rarefaction curves for EM fungal species at roots of girdled (G) and control (C) beech trees. Rarefaction was performed by EcoSim software, using pooled data from all sampling times. Dashed curves represent 95% confidence intervals.



Supplement 5.4

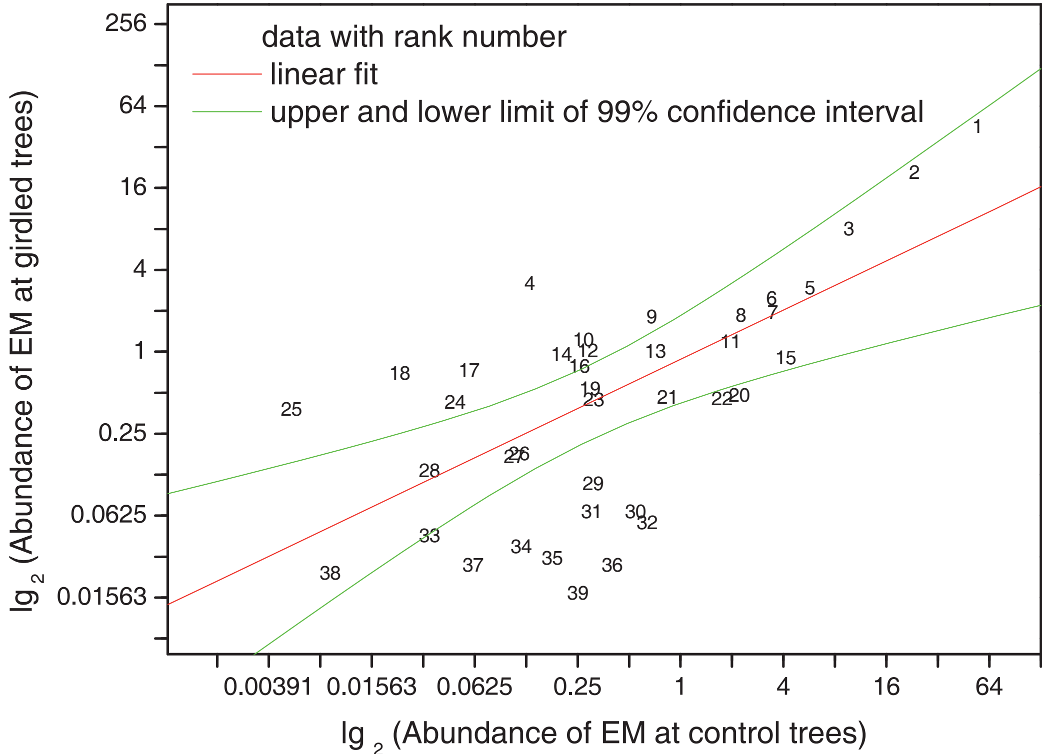
Distribution of beech EM fungi in root samples from girdled (G) and control (C) trees collected in October 2006, May, August and October 2007. Chi square test was used for statistical analysis of deviation of the dispersion index (ID) from a random (Poisson) distribution (ID = 1).

				Critical Values for Chi-square		
Site	Dispersion	ID*	χ^2	0.975		0.025
C- October 06	Random	1.194	16.72	5.628	-	26.1
G- October 06	Uniform	0.974	2.923	< 5.628		
C- May 07	Random	0.433	6.066	5.628	-	26.1
G- May 07	Random	0.46	13.25	5.628	-	26.1
C- August 07	Uniform	0.389	5.459	< 5.628		
G- August 07	Uniform	0.216	3.034	< 5.628		
C- October 07	Random	0.745	10.432	5.628	-	26.1
G- October 07	Random	0.584	8.186	5.628	-	26.1

*ID = σ^2 / μ, where σ^2 = variance and μ = mean; χ^2 = ID (n-1), where n = number of samples = 15 and 3 for G- October 06.

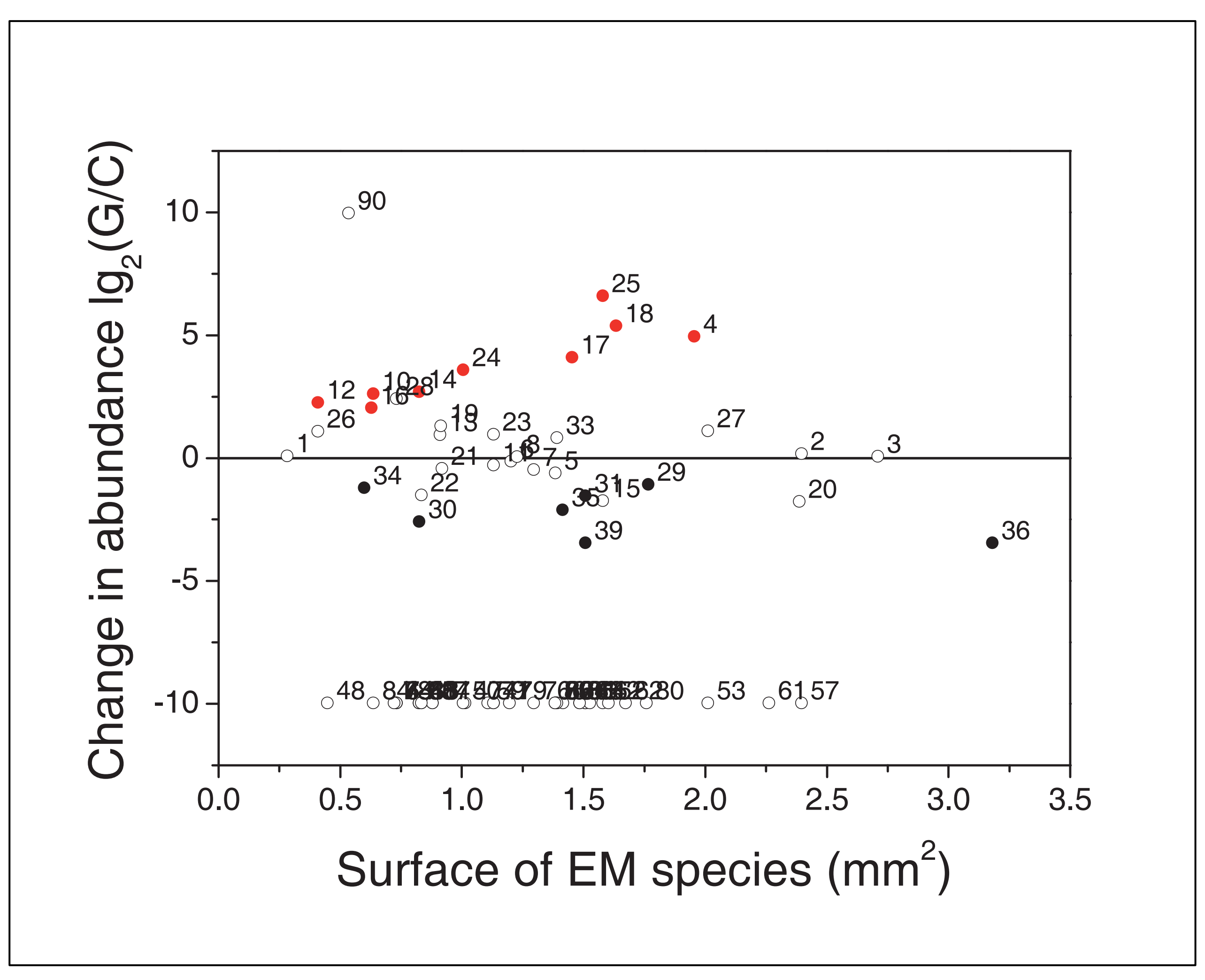
Supplement 5.5

Correlation of the abundance of ecomycorrhiza species at roots of controls with those at root of girdled trees. The 39 species present at roots of both girdled and control trees were included. Data were log2 transformed. Labels at data points indicate rank number according to figure 3 in the main manuscript. Data points outside the confidence range of the linear regression were considered to represent species that were increased or decreased due to the girdling treatment.



Supplement 5.6

Change in EM abundance after girdling in relation to the estimated surface of the EM species. Numbers in the figure refer to rank order according to Figure 3 and Table 3. The change in abundance was calculated as Lg2[(abundance after girdling)/(abundance of controls)]. The surface of EM species was calculated by measuring the diameter (d) and length (l) of the mycorrhizal root tip on photos (see supplement 1) together with a scale bar and assuming a cyclindrical surface (l*d*P+(d/2)2*P). Species that increased (red dots) and decreased (black dots) according to supplement 4 are indicated. For calculation all species that disappeared were set as G/C = 0.001 (rank positions >39<90) and that appeared on girdled trees were set as G/C = 1000 (rank 90)



Supplement 5.7

Pearson product monumentum. Calculation are based on means per sampling date and treatment (n = 8)

Only those fungi were included that were present in at least half of the samples.

Parameter: R = Regression coefficient, P = Probability

Species names and UEM (uncultured ectomycorrhizal fungus) refer to species identified by ITS sequencing, MT = morphotype (non-sequenced species).

Species	Parameter	*C. geophilum*	*L.blennius*	*T. lapida*	UEM 2	UEM 3	*T.lilacinogrisea*	UEM (Hebeloma)	*T.subclavigera*	UEM 4	*I.hirtella*	*X.pruinatus*	*R. amairei*	MT 44	*M. macrosporus*
L. blennius	R	0,319													
	P	0,4412													
T. lapida	R	-0,2024	-0,0264												
	P	0,6307	0,9504												
UEM 2	R	-0,8532	-0,7083	0,2765											
	P	0,0071	0,0493	0,5074											
UEM 3	R	0,1949	0,5157	0,1351	-0,3139										
	P	0,6437	0,1908	0,7498	0,449										
T.lilacinogrisea	R	-0,9058	-0,3676	0,4748	0,8228	-0,3436									

	P	0,0019	0,3703	0,2345	0,0121	0,4047									
UEM (Hebeloma)	R	0,4413	0,2704	0,2228	-0,2797	0,4828	-0,5079								
	P	0,2737	0,5172	0,5959	0,5022	0,2256	0,1987								
T. subclavigera	R	-0,3732	-0,2174	-0,3673	0,1588	-0,6209	0,3205	-0,7169							
	P	0,3626	0,6051	0,3708	0,7073	0,1004	0,439	0,0454							
UEM 4	R	0,2637	-0,2404	-0,2819	-0,0685	0,0919	-0,4046	0,522	-0,1281						
	P	0,5281	0,5663	0,4988	0,8719	0,8287	0,3201	0,1845	0,7625						
I.hirtella	R	-0,1296	0,0607	0,1326	0,0456	0,0716	0,064	-0,1402	0,2111	-0,5896					
	P	0,7597	0,8865	0,7543	0,9147	0,8661	0,8804	0,7405	0,6157	0,124					
X.pruinatus	R	-0,4418	-0,1128	0,2247	0,4374	0,6397	0,3013	-0,0371	-0,3445	-0,1961	0,2621				
	P	0,2731	0,7903	0,5927	0,2784	0,0876	0,4684	0,9304	0,4033	0,6416	0,5305				

R.mairei	R	0,3816	-0,1911	-0,7145	-0,2449	-0,3236	-0,3958	-0,2178	0,0967	0,3672	-0,6255	-0,3948			
	P	0,351	0,6503	0,0464	0,5589	0,4342	0,3317	0,6044	0,8198	0,3709	0,0972	0,3331			
MT 44	R	0,4385	-0,5578	0,0686	0,0471	-0,1644	-0,2405	0,1208	-0,4444	0,2188	-0,3139	-0,0379	0,4783		
	P	0,2771	0,1508	0,8718	0,9118	0,6972	0,5662	0,7757	0,27	0,6027	0,449	0,929	0,2306		
M. macrosporus	R	-0,3653	-0,4791	0,525	0,419	-0,4302	0,5604	-0,2468	0,4582	0,0816	0,0161	-0,1537	-0,3531	-0,0462	
	P	0,3736	0,2296	0,1815	0,3015	0,2874	0,1485	0,5557	0,2536	0,8476	0,9698	0,7164	0,391	0,9134	
T. pilosa	R	-0,5812	-0,6353	-0,1165	0,751	-0,3065	0,4274	-0,2811	0,2276	-0,2149	0,485	0,3919	-0,1534	0,0619	0,03
	P	0,1308	0,0905	0,7835	0,0318	0,4602	0,2909	0,5	0,5877	0,6093	0,2231	0,337	0,7169	0,8842	0,9439

Supplement 5.8

Sørensen's index of similarity (β-diversity) for beech EM fungal community composition in root samples from girdled (G) and control (C) trees collected in May, August and October 2007.

	G – Oct	G – Aug	G – May	C – Oct	C - Aug
C- May	0.423	0.462	0.704	0.472	0.500
C- Aug	0.500	0.783	0.474	0.356	
C- Oct	0.519	0.265	0.458		
G- May	0.391	0.485			
G- Aug	0.452				

Supplement 6.8

Presence of ectomycorrhizal fungal species at roots of healthy beech trees (C) and girdled trees (G) at each sampling date.

Species	C T1	T2	T3	T4	G T1	T2	T3	T4
Cenococcum geophilum	+	+	+	+	+	+	+	+
Lactarius blennius	+	+	+	+	+	+	+	+
Tomentella lapida	+	+	+	+	-	+	+	+
Hysterangium nephriticum	-	+	+	+	+	+	+	-
UEM 2	-	+	+	+	-	+	-	+
Tomentella lilacinogrisea	-	+	+	+	-	-	-	+
UEM (Hebeloma)	+	+	+	+	-	+	+	-
UEM Sebacinacea	+	+	-	-	+	-	-	-
UEM(Tricholoma)	-	+	-	-	+	-	-	-
Tuber puberulum	-	+	-	+	-	+	-	-
Tomentella subclavigera	+	+	+	+	+	-	+	+
UEM 12	-	-	-	+	-	-	-	+
Tomentella viridula	-	+	-	+	-	-	-	+
UEM 4	+	+	+	+	+	+	+	+
Inocybe hirtella	+	+	+	+	-	+	+	+
Sebacina incrustans	+	-	-	-	-	-	-	-
UEM 5	+	-	-	+	-	+	-	-
UEM 6	+	-	-	+	-	-	-	+
UEM 10	+	-	-	+	-	+	-	-
Inocybe pseudoreducta	+	+	-	-	-	+	-	-
Lactarius pallidus	+	-	-	+	-	-	-	-
Russula cuprea	-	-	-	+	-	-	-	-
Xerocomus pruinatus	-	+	+	+	-	+	+	+
Cortinarius boulderensis	+	-	-	-	-	-	-	-
UEM 7	+	-	-	+	-	-	-	-
Russula mairei	+	+	-	+	+	+	-	-
UEM 8	-	+	-	-	-	+	-	-
UEM 11	-	-	-	+	-	-	-	+
MF 105	-	+	-	+	-	+	-	-
MF 44	+	-	-	+	-	+	-	+
MF 67	-	-	-	+	-	+	-	-
Melanogaster macrosporus	+	+	-	+	-	+	-	+
MF 120	-	-	-	+	-	-	-	-
Tomentella pilosa	+	+	-	+	-	+	-	-
MF 101	-	+	-	-	-	-	-	-
Cortinarius solis-occasus	-	-	-	-	-	+	-	-
UEM Russulacea	+	-	-	+	-	-	-	+
Russula foetans	-	-	-	+	-	-	-	-
MF 36	+	-	-	+	-	-	-	-
MF 58	+	-	+	+	-	-	-	-
MF119	+	-	-	+	-	-	-	-
MF 122	+	-	-	+	-	-	-	-
MF 62	+	-	-	+	-	-	-	+
MF 130	-	-	+	-	-	-	-	-
MF 15	+	-	-	+	-	-	-	-
MF 100	-	+	-	+	-	-	-	-
UEM (Genea)	-	+	-	-	-	+	-	-
MF 6	+	-	-	+	-	-	-	-
Humaria hemisphaerica	-	+	-	+	-	-	-	-
MF 126	-	-	-	+	-	-	-	+

Appendix 1

Tree girdling provides insight in the role of labile carbon in the competitive balance of N partitioning between soil microorganisms and adult European beech.

Dannenmann M., Simon J., Gasche R., Holst J., Naumann P. S., Kögel-Knabner I., Knicker H., Mayer H., Schloter M., Pena R., Polle A., Rennenberg H. and Papen H.

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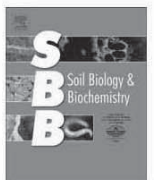

Tree girdling provides insight on the role of labile carbon in nitrogen partitioning between soil microorganisms and adult European beech

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A B S T R A C T

Nitrogen (N) cycling in terrestrial ecosystems is complex since it involves the closely interwoven processes of both N uptake by plants and microbial turnover of a variety of N metabolites. Major interactions between plants and microorganisms involve competition for the same N species, provision of plant nutrients by microorganisms and labile carbon (C) supply to microorganisms by plants via root exudation. Despite these close links between microbial N metabolism and plant N uptake, only a few studies have tried to overcome isolated views of plant N acquisition or microbial N fluxes. In this study we studied competitive patterns of N fluxes in a mountainous beech forest ecosystem between both plants and microorganisms by reducing rhizodeposition by tree girdling. Besides labile C and N pools in soil, we investigated total microbial biomass in soil, microbial N turnover (N mineralization, nitrification, denitrification, microbial immobilization) as well as microbial community structure using denitrifiers and mycorrhizal fungi as model organisms for important functional groups. Furthermore, plant uptake of organic and inorganic N and N metabolite profiles in roots were determined.

Surprisingly plants preferred organic N over inorganic N and nitrate (NO_3^-) over ammonium (NH_4^+) in all treatments. Microbial N turnover and microbial biomass were in general negatively correlated to plant N acquisition and plant N pools, thus indicating strong competition for N between plants and free living microorganisms. The abundance of the dominant mycorrhizal fungi *Cenococcum geophilum* was negatively correlated to total soil microbial biomass but positively correlated to glutamine uptake by beech and amino acid concentration in fine roots indicating a significant role of this mycorrhizal fungus in the acquisition of organic N by beech. Tree girdling in general resulted in a decrease of dissolved organic carbon and total microbial biomass in soil while the abundance of *C. geophilum* remained unaffected, and N uptake by plants was increased. Overall, the girdling-induced decline of rhizodeposition altered the competitive balance of N partitioning in favour of beech and its most abundant mycorrhizal symbiont and at the expense of heterotrophic N turnover by free living microorganisms in soil. Similar to tree girdling, drought periods followed by intensive drying/rewetting events seemed to have favoured N acquisition by plants at the expense of free living microorganisms.

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1. Introduction

Belowground nitrogen (N) cycling in terrestrial ecosystems is characterized by a variety of N transformation processes and fluxes involving both organic and inorganic N species mediated by plants and microorganisms. While the general ecological significance of

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the N cycle in terms of regulating ecosystem N retention, N loss (which can affect atmospheric chemistry, climate change and water quality), and plant nutrition has remained unchanged, our perception of the functioning of this complex network of closely interlinked processes has undergone considerable alterations in recent years (Schimel and Bennett, 2004; Chapman et al., 2006; Van der Heijden et al., 2008). Major paradigm shifts include that (1) the term N mineralization was extended to the step of depolymerisation of organic macromolecules to largely bioavailable monomers (Schimel and Bennett, 2004); (2) plants may take up these organic monomers (e. g. amino acids) and not exclusively inorganic N (Lipson and Näsholm, 2001); (3) plants actively compete for N (organic and inorganic species) with microbes and are not *a priori* inferior in this competition (Schimel and Bennett, 2004); and (4) the fact that not only microorganisms control plant growth but plants may actively control N conversion in soils by microbes (Paavolainen et al., 1998; Erickson et al., 2000; Chapman et al., 2006; Van der Heijden et al., 2008; Zakir et al., 2008).

By successfully competing for N, plants may regulate the N substrate availability for free living microorganisms (Van der Heijden et al., 2008). Competition for N might take place at several "levels" of the N cycle (Schimel and Bennett, 2004): (1) Plants may take up amino compounds, thus limiting this substrate for microbial uptake of dissolved organic nitrogen (DON) and potential subsequent ammonification. (2) Plants may take up NH_4^+, thus reducing substrate availability for autotrophic nitrification and microbial immobilization. (3) Plants may take up nitrate (NO_3^-), thus limiting denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and microbial immobilization of NO_3^-.

Another major influence of plants on microbial N turnover in soil is labile C supply to microorganisms via root exudation. Parts of the carbohydrates produced by photosynthesis in the shoots of plants are transported via the phloem belowground and released into the soil as root exudates. Rhizodeposition constitutes a major source of labile C for heterotrophic microbial processes (Högberg and Read, 2006).

Despite the close link of microbial N metabolism and plant uptake of N, most of the studies performed so far have investigated either the microbial or the plant part of N turnover. Studies interlinking both sides are still rare (Rennenberg et al., 2001; Harrison et al., 2007, 2008), which makes an ecosystematic view of N cycling in the soil impossible. Furthermore, earlier studies on plant–microbe interactions were mostly simple tracer studies which allowed only medium- to long-term determination of ^{15}N recovery rates, but did not investigate plant–microbe competition for N in a process-oriented way.

Girdling of trees, i.e. the removal of a ring of bark and cambium around the stem, interrupts the transport of labile C compounds from the shoots to the roots as well as root exudation (Zeller et al., 2008). Thus, girdling might help to explain the role of the plant to regulate microbial N metabolism. Tree girdling has been reported to result in a decrease of heterotrophic microbial activity in soil (Högberg and Read, 2006). However, after a certain period, roots might die because of exhaustion of their C reserves. This may lead to an increase in N mineralization by microorganisms (Zeller et al., 2008).

In this study, we investigated short-term (6 weeks) and medium-term (14 months) effects of tree girdling on key processes of microbial N turnover (ammonification, nitrification, immobilization of inorganic N), soil C and N pools including microbial biomass, abundance of important microbial groups like mycorrhizal fungi and denitrifiers, plant uptake of both inorganic N and selected amino acids as well as N metabolite profiles in the roots in a temperate beech forest ecosystem. The overall aim of our work was to give a comprehensive insight on the functioning of belowground N partitioning between plants and microorganisms using tree girdling as an experimental approach. In particular, we tested the following hypotheses: (1) Belowground N fluxes in the investigated ecosystem are mediated by competition between plants and soil microorganisms. (2) Beech prefers inorganic N over amino compounds. (3) Tree girdling initially reduces labile C supply to free living soil microorganisms and thus shifts the competitive balance of N partitioning in favour of beech and/or its associated fungal mycorrhizal symbionts.

2. Materials and methods

2.1. Site characteristics

The study site is located in the Swabian Jura, a low mountain range in southern Germany (Tuttlingen Research Station, longitude 8° 45′E; latitude 47°59′N) at an altitude of 800 m a.s.l.. Mean annual air temperature is approximately 6.5 °C and the average annual precipitation amounts to 854 mm (1961–1990). The study site is exposed to a comparatively low atmospheric N deposition of <10 kg N ha^{-1} $year^{-1}$ (Dannenmann et al., 2008). The experimental site of the present study was implemented on the steep slope (approx. 25° inclination) of a narrow valley facing northeast, at a distance of approximately 300 m from a long-term ecological research site equipped with a meteorological tower ("NE" or "N" site, see Geßler et al., 2001; Holst et al., 2004, 2005; Dannenmann et al., 2006, 2007a,b, 2008). Both altitude and exposure of the long-term site and the newly established girdling experiment site are identical. The soil was classified as Rendzic Leptosol (Sceletic) (IUSS Working Group BWB, 2006) derived from horizontally bedded limestone and marls. Soil profiles are shallow followed by weathered parent rock containing >45% gravel and stones. The Ah horizon at the girdling site is characterized by high organic C content (11.0 ± 0.4%), high N content (0.8 ± 0.1%), a C/N ratio of 13.2 ± 0.2 and a pH value of 6.8 ± 0.05. The clay content of the soil is high (70.1 ± 1.4%), while silt (26.8 ± 1.8%) and sand (3.2 ± 1.1%) contributed to soil texture to a minor degree. Beech (*Fagus sylvatica* L.) is the dominant species contributing >90% of the total basal area of adult trees. Nearly all fine roots are colonized by mycorrhizal fungi.

2.2. Meteorological data

Soil temperature and volumetric soil moisture were recorded every 30 s and stored as 10 min mean values, which were finally aggregated to daily mean values. Soil temperature was measured at 3 cm depth by use of a PT100 probe. Soil moisture was determined using two probes according to the time domain reflectometry method (TDR, CS615, Campbell Sci., Shepshed, GB). The probes, which have a sensor length of 0.2 m, are buried vertically in the ground, thus covering the uppermost 0.2 m of the slope-parallel ground. The TDR soil moisture recordings were compared with gravimetrically taken soil moisture measurements to allow a soil-specific calibration. Soil moisture used represents the mean value from both probes.

2.3. Experimental design

In August 2006, a uniform slope patch of 27° angle at middle slope position was selected as the site for the current experiment. The maximum altitude difference at the selected site is approximately 20 m. Within this site, six experimental plots each consisting of 5 adjacent adult beech trees were created. The plot size was in the range of approximately 30–48 m^2. Trees of three randomly selected plots were girdled on 9 August, 2006 while three

plots remained untreated. Girdling was performed by removing bark and cambium of trees approximately 1.2 m above ground. The height of the concentric circle of the girdled area was 8.3 cm. Sampling was performed at the end of the vegetation period when rhizodeposition peaked.

2.4. Soil and plant material sampling

Sampling was performed on 10 October, 2006 and 25 September, 2007. For this purpose, five soil pits were dug at each plot at both sampling periods. All soil pits were located slope-parallel at a distance of 1 m to a tree. The soil pits were approx. 0.4 m deep and revealed soil profiles of the complete Ah horizon. Soil samples were taken from the very densely rooted uppermost soil layers (0–0.2 m depth) which corresponded mostly to the entire Ah horizon. Soil was taken from the profile using small shovels as several intact soil portions of approximately 500 cm^3 at each pit. Soil samples of the five pits per plot were composited for analyses of gross rates of microbial N turnover and microbial biomass C and N as well as abundance of denitrifiers and extractable dissolved organic carbon (DOC) and dissolved organic nitrogen (DON). Samples were immediately stored until further analysis at 4 °C except samples for molecular analysis of denitrifiers, which were stored at −80 °C. All soil samples stored at 4 °C were extracted within 1 week after sampling.

Simultaneous with soil sampling, root material was collected from mature beech trees. All plots were sampled for root material of five trees each during both sampling campaigns. Root material was carefully dug out of the soil, washed with tap water to remove adhering soil particles and immediately frozen in liquid N_2. Samples were stored at −80 °C on return from the field.

2.5. N uptake by the roots

Net N uptake in fine roots of adult beech trees was examined using the ^{15}N enrichment technique as described by Geßler et al. (1998). The root tips still attached to the trees were incubated for 2 h (from 10 am to 12 pm) in 4 ml of an artificial soil solution of similar pH and concentrations of inorganic anions and cations as in the soil water at the site (Geßler et al., 2005). The concentrations of N-containing compounds were 10 μM NO_3^- (as KNO_3), 0.1 μM NH_4^+ (as NH_4Cl) and 2.5 μM of glutamine (Gln) and arginine (Arg) each. Gln and Arg were chosen as the most abundant amino compounds of beech. The artificial solutions contained ^{15}N-label either as ^{15}N–NO_3^-, ^{15}N–NH_4^+ or $^{15}N/^{13}C$-double labelled Gln/Arg. In addition, control solutions with no ^{15}N-label were used to account for the natural abundance of ^{15}N in the roots. The soil temperature at 5 cm depth during the uptake experiments in 2006 and 2007 was, on average, 10 °C and 9 °C, respectively. The calculation of net uptake rates (nmol g^{-1} fw h^{-1}) was based on the incorporation of ^{15}N into root fresh weight.

2.6. Quantitative chemical analysis in fine and coarse roots

For all chemical analyses, frozen root material was ground to a fine powder with a mortar and pestle in liquid N_2 and kept at −80 °C until further analysis.

2.6.1. Total N and $\delta^{15}N$ ratio

Total N concentration and $\delta^{15}N$ ratio of oven-dried (48 h, 60 °C) root material (0.5–1.0 mg) were determined using an elemental analyzer (NA 2500, CE Instruments, Milan, Italy), coupled with a Conflo II interface to an isotope ratio mass spectrometer (Delta Plus, Finnigan MAT GmbH, Bremen, Germany). Isotope ratios are expressed as delta notation (in ‰), relative to the air standard.

2.6.2. Total soluble protein

For total soluble protein analysis, approximately 0.05 g of frozen root tissue was extracted in 1 ml of buffer (50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 15% glycerol (v:v), phenylmethylsulfonylchloride (PMSF, 1 mM), dithiothreitol (DTT, 5 mM) and 0.1% Triton X-100) by vortexing and incubation for 30 min at 4 °C. After centrifugation at 14,000 × g for 10 min at 4 °C, 500 μl aliquots of the supernatant were transferred to new tubes. Five hundred microlitres of trichloroacetic acid were added, and samples were incubated for 10 min at room temperature. After centrifugation (14,000 × g, 10 min at 4 °C) the pellet was dissolved in 1 ml of 1 M KOH. For quantification of soluble protein, 1 ml of Bradford Reagent (Amresco Inc., Solon, Ohio, USA) was added to 50 μl aliquots. After 10 min incubation at room temperature in the dark, the optical density was measured in a UV-DU650 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA) at 595 nm. Bovine serum albumin (BSA, Sigma A-6918) was used as a standard.

2.6.3. Amino compounds and NH_4^+

Amino compounds and NH_4^+ were extracted according to the method of Winter et al. (1992). Extracts were shock-frozen in liquid N_2 and freeze-dried for 96 h. Amino compound composition and concentration were determined in 50 μl samples using a Waters Acquity UPLC-System (Waters Corp., Milford, MA, USA) with a modified standard protocol (using an AccQ-Tag™ Ultra column 2.1 × 100 mm, 1.7 μm, 0.7 ml/min flow, column temperature 61 °C). Amino acid Standard H (#NCI0180, Pierce Biotechnology, Inc., Rockford, IL, USA) was used as an analytical standard. Additional amino acids were added (with 2.5 μmol in 0.1 N HCl each) according to the composition of the samples analysed.

2.6.4. Nitrate

For the extraction of anions, 0.1 g of washed PVP was soaked in 1 ml of distilled water for 2 h before adding 0.05 g of root material to the mixture. After shaking in the dark for 1 h at 4 °C to bind phenols, samples were boiled at 95 °C for 10 min and centrifuged (14,000 × g, 10 min at 4 °C). Aliquots of the supernatants were analyzed using an ion chromatograph (DX 120, Dionex, Idstein, Germany) combined with an autosampler (AS 3500, Thermo Separation Products, Piscataway, USA) and equipped with the PeakNet software package (version 4.3, Dionex, Idstein, Germany). Separation of anions was achieved using an eluent solution of 2.0 mM sodium carbonate and 0.75 mM sodium bicarbonate. Anion mixtures of NO_3^-, PO_4^{3-}, SO_3^{2-} and SO_4^{2-} in dist. H_2O were used as standards.

2.7. Gross rates of microbial N turnover, inorganic N concentrations

Gross rates of ammonification and nitrification were determined using the ^{15}N pool dilution technique as described in detail by Dannenmann et al. (2006) for identical soil. Two days before the start of the experiment, the soil samples were pre-incubated at the experimental incubation temperature (10 °C in 2006 and 9 °C in 2007). Immediately before the start of the experiment, gravel, stones and roots were removed from the soil by carefully breaking the intact soil sample portions by hand prior to sieving (5 mm mesh width). Mechanical disruption of the soil was minimized as far as possible. Two subsamples (230 g sieved soil each) were labelled with 7 ml of 30% ^{15}N-enriched KNO_3 solution (for determination of gross nitrification rates) or 7 ml of 30% ^{15}N-enriched $(NH_4)_2SO_4$ solution (for determination of gross ammonification rates), respectively (time t_0). The subsamples were spread in a thin layer and then the ^{15}N label solution was sprayed homogenously on the samples. The amount of added N corresponded to 1 μg N g^{-1} sdw. While aliquots of 180 g of the subsamples were transferred into six 250 ml plastic bottles (Carl Roth GmbH, Karlsruhe, Germany) (30 g

each), the residual soil was used for determination of the gravimetric water content. The plastic bottles were incubated in the dark. At time t_1 ($= t_0 + 24$ h) and time t_2 ($= t_0 + 48$ h) soil in three of the bottles was extracted with 1 M KCl (Dannenmann et al., 2006). Subsamples of the filtrate were passed through 0.45 μm syringe-filters (Schleicher & Schuell, Dassel, Germany) and immediately frozen until colourimetric measurement of NH_4^+ and NO_3 concentrations by a commercial laboratory (Dr Janssen, Gillersheim, Germany). The diffusion method (Brooks et al., 1989) was used for sequentially trapping NH_4^+ and NO_3 as NH_3 on acidified ashless paper filters as described by Dannenmann et al. (2006). The $^{14}/^{15}$N-ratio of the N captured on the dried filter papers was analyzed using an elemental analyzer (EA 1110, Carlo Erba Instruments, Milan, Italy) coupled to a mass spectrometer (MAT Delta Plus, Thermo Finnigan, Bremen, Germany). Gross ammonification and gross nitrification rates were calculated using the equations given by Kirkham and Bartholomew (1954). Microbial immobilization of NH_4^+ was estimated by subtracting the nitrification rates from NH_4^+ consumption rates while NO_3 consumption was assumed to equal microbial NO_3 immobilization (Davidson et al., 1991).

For determination of the NH_4^+ and NO_3 contents, 30 g of unlabelled soil free of limestone and roots was extracted with 1 M KCl solution as described above.

2.8. Soil texture, organic C and total N content, pH values

Analysis of soil texture and C and N contents were conducted according to the methods described by Mueller and Koegel-Knabner (2009). Due to the presence of carbonates, soil samples were treated with 1 N HCl prior to analysis. Inorganic C concentrations of samples were determined after ignition of samples for 3 h at 550 °C. The organic C concentrations of the samples were calculated as the difference between total and inorganic C. Soil pH values (0.01 M $CaCl_2$) were determined as described by Dannenmann et al. (2006).

2.9. Total microbial biomass C and N, extractable DOC and DON in soil

Microbial biomass C and N was estimated using the chloroform fumigation–extraction (FE) method (Brookes et al., 1985; Vance et al., 1987) as described in detail by Dannenmann et al. (2006, 2007b). Samples were divided in paired subsamples of 30 g each. One subsample was immediately extracted with 60 ml of 0.5 M K_2SO_4 while the second subsample was fumigated under chloroform vapour for 24 h in a desiccator. Subsequently, 10 vacuum/release purge cycles ensured the complete removal of chloroform and fumigated subsamples were extracted as described above. Extracts were filtered using a 0.45 μm syringe filter (Schleicher & Schuell, Dassel, Germany) and immediately frozen until analysis for total organic carbon (TOC) and total chemically bound nitrogen (TNb) using a TOC analyzer with a coupled TNb module as described by Dannenmann et al. (2006). Correction factors (0.54 for microbial biomass N and 0.379 for microbial biomass C; Brookes et al., 1985; Vance et al., 1987) were applied to the difference in total extractable N and TOC between paired untreated and fumigated subsamples to estimate microbial biomass C and N. TOC concentrations as obtained from the untreated control samples are referred to as extractable DOC while extractable DON was calculated by subtracting DIN (dissolved inorganic N, calculated as NH_4^+ concentrations + NO_3 concentrations) from TNb.

2.10. Quantification of the denitrifying bacteria

DNA was extracted from 0.5 g of soil according to the protocol described by Griffiths et al. (2000) and slightly modified by Sharma et al. (2005). The method principally involved bead beating and solvent extraction of the nucleic acids. Quality and quantity of the DNA extracts were checked with a spectrophotometer (Nanodrop, PeqLab, Germany). Extracts were stored at −20 °C until use.

Quantitative real-time PCR was conducted on a 7300 Real-Time PCR System (Applied Biosystems, Germany) using SybrGreen as fluorescent dye. Details about PCR conditions are described by Babic et al. (2008). In a pre-experiment, dilution series of the different DNA extracts were tested to avoid inhibition of PCR, e.g. by co-extracted humic substances. DNA extract dilutions of 1:30 turned out to be best suited for this purpose (data not shown). Amplifications were performed in 96-well plates, conducted in triplicate for all standards, non-template controls, and samples. After each PCR run, a dissociation curve was attached consisting of 95 °C for 15 s, 60 °C for 30 s, and a subsequent temperature increase until 95 °C with a ramp rate of 0.03 °C s^{-1}. Purity of amplified products was checked by the observation of a single melting peak and the presence of a unique band of the expected size in a 1.5% agarose gel stained with ethidium bromide. The amplification efficiencies were calculated from the formula $Eff = [10^{(-1/slope)} - 1]$.

2.11. Abundance of Cenococcum geophilum

Soil cores with a volume of 1 l (0.08 m diameter, 0.2 m height) were removed at a distance of about 1 m from the stem at each of the 15 girdled and 15 control trees. In October 2006 for girdled plots only, one mixed sample containing pooled soil cores from the three girdled plots was obtained. The soil cores were transported to the laboratory and stored at 4 °C until analysis. The roots were carefully washed out of the soil. They were mixed and four to six roots of each sample were studied under a stereomicroscope (Stemi SV 11, Zeiss, Jena, Germany). The number of mycorrhizal root tips was counted and the frequency of *C. geophilum*, which was the dominant ectomycorrhizal species and which can easily be recognized by its typical black appearance, was noted. For identification the key of Agerer (1987–2006) was used. The identity of *C. geophilum* was also checked by ITS sequencing of collected morphotypes (Pena and Polle, unpublished) and the sequence was deposited at GenBank under accession number FJ403485.

2.12. Statistics

Correlation and regression analyses were performed based on plot means of the measured parameters for single sampling dates ($N = 6$ if not otherwise stated). Furthermore, Pearson's correlation coefficients were calculated. Effects of the treatment (girdling versus untreated control) and variation between sampling dates (2007 versus 2006) were tested using the Mann–Whitney U-test ($n = 3$ per treatment and sampling date). The threshold value for significant correlations or differences was set at $p < 0.05$. All statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, USA).

3. Results

3.1. Meteorological data

The meteorological conditions before the two sampling dates of this study were different. Extremely dry soil conditions were observed in 2006 from June to July, followed by intense drying/rewetting cycles from August to the sampling date. Soil moisture in July 2006 was one of the lowest values determined since the start of the measurements in 2000. In contrast, drying/rewetting events generally occurred at higher soil moisture level and lower amplitude in 2007, while there was no pronounced drought period in the 4 months before sampling.

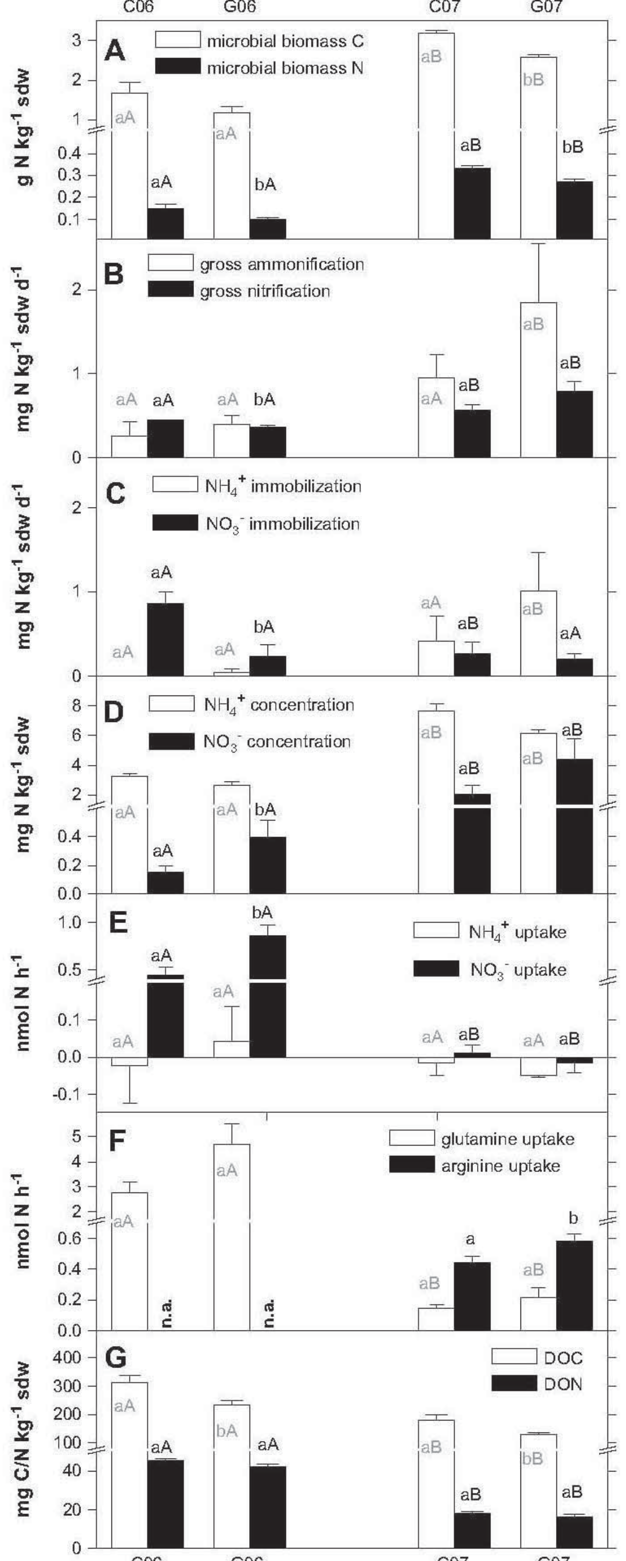

Fig. 1. Mean biomass C and N (A); gross rates of microbial N turnover (B, C); extractable inorganic N concentrations in soil (D); plant uptake of inorganic N (E) and amino acids (F); and DOC/DON concentrations in soil for the two experimental treatments (control, girdled) and the two sampling dates (September 2006; September 2007). Means and standard errors were calculated from $N = 3$ replicated plots per treatment. C, control plots; G, girdling plots; 06, 07, year of sampling date. Different lower case letters indicate significant differences between C and G at the same sampling date while different capital letters indicate significant differences between the sampling dates at a given treatment.

However, due to rainfall immediately before sampling in 2006, the gravimetric soil water content was significantly higher on October 10th 2006 (control plots: 66.8 ± 1.7% sdw; girdled plots: 60.9 ± 1.9% sdw) than on September 25th 2007 (control plots: 56.0 ± 2.4% sdw; girdled plots: 51.5 ± 2.5% sdw). Thus, the water content of the sampled soil is not representative for the weeks and months before sampling. For both years, soil water content at the girdled plots was slightly, but significantly, lower compared to the control plots.

3.2. Total microbial biomass and microbial community structure

Both microbial biomass C and N pools were significantly larger in 2007 than in 2006 (Fig. 1A, $p < 0.05$). Despite different magnitudes of pool sizes in 2006 and 2007, a consistent pattern towards smaller microbial biomass pool sizes at girdled plots than at control plots was observed for both sampling dates (Fig. 1A). These treatment effects on microbial biomass C and N were significant for microbial biomass N in both years ($p < 0.05$) and for microbial biomass C in 2007 ($p = 0.05$) (Fig. 1A).

As an example for the influence of the girdling on microbial community structure two major functional groups were characterized (denitrifying microbes and mycorrhizal fungi). Girdling resulted in reduced numbers of nitrous oxide reductase genes (*nosZ*), which was used as a marker for denitrifiers in soil compared to the control (1818 ± 305 copies ng^{-1} at control plots and 1571 ± 167 copies ng^{-1} DNA at girdled plots; data only available for 2006). However, due to the high standard error in the control samples, this effect was not statistically significant. Interestingly, the ratio between microbial biomass C and N and *nosZ* gene abundance was constant in all samples, indicating that denitrifying microbes were influenced by the girdling, similar to other functional groups of bacteria and fungi.

Girdling affected the ectomycorrhizal community composition (Pena et al., submitted for publication), but not the overall abundance of *C. geophilum*, which was the most frequently observed EM fungus at root tips (Table 2). The abundance of *C. geophilum* was higher in samples from 2006 compared to samples from 2007 (Table 2).

3.3. DOC and DON in soil

While DON concentrations were not significantly different between adjacent control and girdled plots, DOC concentrations were significantly lower at the girdled plots both in 2006 and 2007 (Fig. 1G). The extractable soil DON pool was of similar size like the extractable DIN pool in 2007 but more than one order of magnitude larger in 2006 (Fig. 1D,G). Both extractable DOC and DON concentrations were significantly smaller in 2007 than in 2006 (Fig. 1G). The ratio between extractable DOC and extractable DON was significantly lower in 2006 than in 2007 and tended to be slightly, but not significantly lower at girdled plots (Table 2).

3.4. Gross rates of microbial N turnover

In 2006, gross rates of ammonification and nitrification were both low (<0.5 mg N kg^{-1} sdw) but considerably higher in 2007

(Fig. 1B). These differences between sampling dates were significant for gross nitrification at both control and girdled plots, but for gross ammonification only at girdled plots (Fig. 1B). There were no significant effects of girdling on gross rates of inorganic N production. In 2006, gross nitrification was of similar magnitude compared to gross ammonification, and NH_4^+ immobilization was not a significant process (Fig. 1B,C). Consequently, NO_3^- served as the only significant inorganic N source for microorganisms in 2006 (Fig. 1B,C). Microbial NO_3^- immobilization was significantly lower at control plots than at girdled plots in 2006. In 2007, gross ammonification rates tended to be higher than gross nitrification rates and gross NH_4^+ immobilization rates were not only a significant process but also higher than gross rates of NO_3^- immobilization (Fig. 1B,C). There were no effects of the girdling treatment on gross rates of microbial production and consumption of inorganic N in 2007.

3.5. Extractable soil inorganic N concentrations

Ammonium was the dominant form of extractable inorganic N in the investigated soil (Fig. 1D). Extractable NH_4^+ concentrations showed a similar pattern as microbial biomass C and N concentrations (Fig. 1A,D). They were significantly higher in 2007 compared to 2006 and tended to be lower in girdled plots than in control plots at both sampling dates (Fig. 1D). However, differences between control and girdled plots were not significant. Soil NO_3^- concentrations were also higher in 2007 than in 2006. In contrast to NH_4^+ concentrations, there was a consistent pattern towards increased NO_3^- concentrations at girdled plots which was significant in 2006.

3.6. Tree uptake of inorganic and organic N

Beech trees preferred amino acids over inorganic N and NO_3^- over NH_4^+ throughout the investigated time span (Fig. 1E,F). There was no significant net NH_4^+ uptake while NO_3^- was assimilated by the trees in significant amounts in 2006, but not in 2007 (Fig. 1E). Gln uptake was higher in 2006 than in 2007. In 2007 trees preferred Arg over Gln. Overall, there was a consistent pattern of higher net N uptake at the girdled plots than at control plots independent of the N species acquired (organic/inorganic; Gln/Arg) (Fig. 1E,F). Net N uptake was significantly higher at girdled plots than at control plots for NO_3^- in 2006 and Arg in 2007. Additionally, both Gln and NO_3^- uptake were significantly higher in 2006 compared to 2007, thus showing opposite patterns between the sampling dates compared to microbial biomass and gross rates of microbial N turnover (Fig. 1A,B,C,E,F).

Based on ^{13}C incorporated into root fresh weight, uptake rates of amino acids were generally significantly lower than based on ^{15}N incorporation. Lower ^{13}C uptake of roots may have been either due to degradation of Gln and Arg in the solution, or further C metabolization in the plant including ^{13}C respiration losses.

3.7. Nitrogen fractions and metabolites in roots

Neither root total N, nor soluble protein, NO_3^- or NH_4^+ contents differed significantly between samples taken from girdled trees and the not girdled controls, both in fine roots at both sampling dates and in course roots (Table 1). This finding indicates that N nutrition of the roots was largely maintained irrespective of girdling. Still, significant differences were found in amino acid composition and contents between treatments and the time of analyses (Table 1). In fine roots in 2006 and course roots in 2007, significantly higher amino acid contents were found in samples of girdled trees compared to the controls. These higher amino acid concentrations were largely attributed to enhanced levels of Arg or/and Asn, both constituting important storage and transport forms of amino N in beech, but also to other amino acids including Val, Leu, Ile, and Thr (Table 1). Amino acid concentrations in the fine roots of controls were significantly higher in 2007 compared to 2006, thereby compensating the girdling effect on fine roots observed in 2006. This increase in fine root amino acid content between 2006 and 2007 was of more general nature, since the majority of the amino acids studied and all metabolic groups of amino acids were affected.

3.8. Correlation analyses between plant and microbial parameters, and DOC concentrations

Glutamine uptake by beech was negatively correlated with soil NH_4^+ concentrations ($R = 0.90$, $p = 0.01$) and gross nitrification in 2006 ($R = -0.88$, $p = 0.02$) while microbial NH_4^+ immobilization was negatively correlated with plant NO_3^- uptake in 2007 ($R = -0.817$, $p = 0.03$). Total microbial biomass was negatively correlated with total amino acid content in fine roots in 2006 ($R = -0.89$, $p = 0.04$) and negatively correlated with arginine concentrations in fine roots ($R = -0.82$, $p = 0.04$) and total soluble protein concentrations in fine roots ($R = -0.86$, $p = 0.03$) in 2007. In contrast, the abundance of *C. geophilum* was positively correlated to asparagine concentrations ($R = 0.901$, $p = 0.01$) and arginine concentrations ($R = 0.81$, $p = 0.05$) in 2007.

Pooling the data of both sampling dates for correlation analyses supported the negative relationship between glutamine uptake by beech and soil NH_4^+ concentrations ($R = -0.90$, $p < 0.001$) as well as between soluble protein concentrations in fine roots and microbial

Table 1
Descriptive statistics for N-metabolites in roots of *Fagus sylvatica* subjected to girdling. Mean and SE mean concentrations are presented for girdled and control plots ($N = 3$ each) in 2006 and 2007. Significant differences between treatments (within fine roots 2006, fine roots 2007 or coarse roots 2007) are indicated with asterisks (*), whereas significant differences between years (only for fine roots) are indicated in bold (for each treatment). Total N, total nitrogen; total AA, total amino acids; Asn, asparagine; Arg, arginine; Thr, threonine; Val, valine; Ile, isoleucine; Leu, leucine.

Variable	Fine roots 2006		Fine roots 2007		Coarse roots 2007	
	Control	Girdling	Control	Girdling	Control	Girdling
Total N (mg g^{-1} dw)	12.64 ± 1.96	12.41 ± 0.75	10.81 ± 0.82	11.10 ± 0.94	5.20 ± 0.09**	5.87 ± 0.21
Soluble protein (mg g^{-1} fw)	**6.75 ± 0.24**	5.91 ± 0.34	**3.96 ± 0.69**	5.91 ± 0.26	5.70 ± 0.41	5.55 ± 0.38
Ammonium (μmol g^{-1} fw)	n. a.	n. a.	0.23 ± 0.10	0.35 ± 0.06	0.39 ± 0.12	n. a.
Nitrate (μmol g^{-1} fw)	**0.31 ± 0.04**	**0.41 ± 0.03**	**0.65 ± 0.07**	**0.55 ± 0.02**	0.76 ± 0.11	0.81 ± 0.27
Total AA (μmol g^{-1} fw)	1.19 ± 0.27*	2.00 ± 0.22	1.55 ± 0.18	1.63 ± 0.45	3.70 ± 0.45*	5.53 ± 0.24
Asn (μmol g^{-1} fw)	0.46 ± 0.29	0.64 ± 0.22	0.26 ± 0.004	0.33 ± 0.07	1.48 ± 0.37	2.76 ± 0.48
Arg (μmol g^{-1} fw)	**0.01 ± 0.03**	0.09 ± 0.05	**0.06 ± 0.003****	0.35 ± 0.19	0.22 ± 0.01**	0.64 ± 0.05
Thr (μmol g^{-1} fw)	**0.03 ± 0.007**	**0.02 ± 0.003**	**0.05 ± 0.002**	**0.05 ± 0.003**	0.04 ± 0.008**	0.07 ± 0.06
Val (μmol g^{-1} fw)	0.02 ± 0.03	0.03 ± 0.007	0.03 ± 0.006	0.02 ± 0.003	0.007 ± 0.002*	0.01 ± 0.001
Ile (μmol g^{-1} fw)	0.006 ± 0.005	0.001 ± 0.005	0.01 ± 0.002*	0.006 ± 0.003	0.008 ± 0.001**	0.02 ± 0.005
Leu (μmol g^{-1} fw)	0.0001 ± 0.0007*	0.02 ± 0.004	0.02 ± 0.008	0.008 ± 0.008	0.005 ± 0.0001	0.01 ± 0.03

biomass N ($R = -0.81$, $p = 0.02$) and indicated further negative relationships between plant and microbial parameters and processes, i. e. negative correlations between glutamine uptake by beech and gross ammonification ($R = -0.58$, $p = 0.05$), gross nitrification ($R = -0.71$, $p =$) and microbial biomass N ($R = -0.91$, $p = 0.01$) as well as between between NO_3 uptake by beech and microbial biomass ($R = -0.859$, $p < 0.001$). Furthermore, combining data from both years for correlation analysis indicated a positive relationship between the abundance of *C. geophilum* and glutamine uptake by beech ($R = 0.67$, $p = 0.04$). However, despite different sampling spots were sampled in the two years, these analyses include data from the same plots but different sampling dates which – from a statistical point of view – might be referred to as not independent. Thus, the explanatory power of these correlation analyses including both year data is limited.

The ratio between microbial biomass N and the soluble protein concentrations in fine roots as an index of competition between microorganisms and beech was positively correlated to soil DOC concentrations in 2007 ($R = 0.82$, $p = 0.04$). DOC was additionally correlated to microbial biomass N in 2007 ($R = 0.84$, $p = 0.04$) and microbial NO_3 immobilization in 2006 ($R = 0.86$, $p = 0.03$).

4. Discussion

4.1. Competitive patterns of N partitioning between plants and microorganisms

The set of negative correlations of microbial N pools and N turnover rates with plant N uptake and N metabolic concentrations in fine roots found within this study indicates that plants and free living microorganisms compete for N as a growth limiting nutrient. High microbial activity may limit plant N acquisition due to the higher substrate affinity, higher surface area to volume ratio, higher growth rates and greater spatial distribution of microorganisms (Hodge et al., 2000). While both trees and free living microorganisms compete for DON at variable levels of success in the investigated ecosystem, soil NH_4^+, in contrast, was primarily immobilized or nitrified by microbes but not taken up by the trees. This is consistent with the finding that net ammonification rates are constantly close to zero or even negative in the investigated ecosystem (Dannenmann et al., 2006). However, NH_4^+ immobilized by free microorganisms might repeatedly return into the "organic competition pool" via dieback of microbial biomass (Harrison et al., 2007). Thus, the long-term competitive strength of plants for N acquisition may be underestimated due to the longer resilience time of N in plants than in microorganisms (Schimel and Bennett, 2004).

Thus, taking up DON partially intact and partially after mineralization to NH_4^+ (e. g. by mycorrhizal symbionts) may be a mechanism of "short-circuiting" N mineralization by free living microorganisms, and thus, of avoiding the strong competition for NH_4^+ (Schimel and Bennett, 2004). In contrast, NO_3 seems to be more readily available in the soil of the investigated forest ecosystem and, thus, taken up by both, trees and free living microorganisms. Negative correlations between microbial biomass N and net NO_3 uptake by trees and positive correlations between DOC and microbial NO_3 immobilization may indicate that microbes compete well for NO_3 in this soil when environmental conditions (especially C supply) are favourable, and thus, limit plant NO_3 uptake.

4.2. Beech prefers amino acids over inorganic N

The preference of beech trees for – partially intact (see below) – amino acids over inorganic N which was found within this study is, with regard to the assumption that this is observed only in strongly N-limited ecosystems, rather surprising. Geßler et al. (2005) found higher NH_4^+ uptake capacity of mycorrhizal beech fine roots than NO_3 uptake capacity in the plots close by, but did not measure amino acid uptake. However, based on the absence of both NH_4^+ in the soil solution and significant net ammonification rates in the soil, it was concluded in the latter study that NO_3 is the main inorganic N source for beech trees at the investigated site. Within this study we could show that – at least during the observation dates – beech trees were able to take up both Arg and Gln at appreciable rates. At high uptake rates observed in 2006, the ratio of ^{13}C:^{15}N incorporation suggests that about half of the amino acids were taken up in intact form (data not shown). The other half could have been mineralized by the mycorrhizal fungal symbionts before provided to the plant, or alternatively respired by the plant. *C. geophilum*, the most abundant mycorrhizal fungi in the investigated ecosystem may play a role in the plant uptake of Gln uptake by beech as indicated by the significant positive correlations to some amino acid concentrations in the roots and Gln uptake. Ectomycorrhizal fungi may help the plant in the competition for N by the greater soil access by hyphae, thus facilitating uptake of slow-diffusing amino acids and probably also by degrading proteins into plant available forms (Jackson et al., 2008). Genome analysis of the ectomycorrhizal fungus *Laccaria bicolor* revealed that almost the whole suite of "saprophytic" genes, i.e. genes encoding enzymes necessary for degradation of organic molecules, were present in this species (Martin et al., 2008). The incorporated amino acids may either result from degradation of SOM or microbial biomass, thus directly contributing to plant nutrition, or from DON losses via root exudation, thus reflecting a recapturing mechanism (Jones et al., 2005). The observation that DON uptake largely exceeded DIN uptake may indicate a significant role of amino acids for tree nutrition in the investigated forest ecosystem. However, due to the limited temporal resolution of our measurements, hypotheses on the quantitative role of DON versus DIN uptake for tree nutrition remain speculative. Nevertheless, the present study widens the range of ecosystems where plant uptake of organic N occurs, since actual uptake of intact amino compounds, or the physiological capacity to take up amino acids, has been observed only in a few temperate forests (Finzi and Berthrong, 2005; Schmidt and Stewart, 1999), but, to our knowledge, not in typical temperate forests of central Europe. The direct uptake of amino compounds by plants has been previously shown for a variety of strongly N-limited ecosystems, e. g. tundra (Chapin et al., 1993; Kielland, 1994; Schimel and Chapin, 1996; Lipson and Monson, 1998; Raab et al., 1999; Henry and Jefferies, 2003; Nordin et al., 2004), boreal forests (Näsholm et al., 1998; Nordin et al., 2001), arid ecosystems (Schmidt and Stewart, 1999) and low-productivity grasslands (Bardgett et al., 2003; Weigelt et al., 2003, 2005; Harrison et al., 2007).

4.3. Girdling shifts the competitive balance of N partitioning in favour of beech and at the expense of free living microorganisms

To evaluate the influence of organic C on competitive interactions between microbial N turnover and plant N uptake, tree girdling was chosen to alter rhizodeposition of easily available C by roots which represents a close link between C and N cycling as well as between plant and microbial processes (Schimel and Bennett, 2004; Bais et al., 2006; Högberg and Read, 2006). Although extractable DON and DOC pools as determined within this study did not exclusively comprise these C and N compounds from root exudation, DOC concentrations but not DON concentrations were depleted in the soil of the girdled plots (Fig. 1G). This is consistent with the assumption that rhizodeposition compounds may claim a major role in soil C fluxes (Högberg and Read, 2006). Effects on soil DOC were already observed a few weeks after girdling was

performed. This comparably fast response of DOC concentrations to the girdling treatment is in line with Högberg et al. (2001) who observed a 37% decrease of soil respiration within five days and a 56% reduction of soil respiration after 14 days following tree stem girdling in a boreal forest.

In the girdling experiment of this study, even more than one year after girdling, root carbohydrates were depleted but not completely exhausted (Pena et al., submitted for publication). Furthermore, there were no clear effects on total N content in fine roots, but considerable effects on metabolite pools were observed (Table 1). Immediately after the girdling treatment, amino acid levels in fine roots increased by enhanced accumulation of storage and transport amino acids (Arg, Asn), an effect that "moved" to the course roots in the subsequent year. This observation suggests that the use of N for root growth was reduced in favour of N storage and transport, an effect that may be expected at reduced C supply to the roots by phloem transport. This view is consistent with the enhanced rates of N uptake by the roots of girdled trees observed in this study (Fig. 1E,F) that still was not sufficiently high to affect total N content of the roots significantly. Higher net N uptake rates may be facilitated by a decrease of rhizodeposition of C compounds leading to lower DOC in the soil of the girdled plots (Fig. 1G), and subsequently to a decrease in microbial biomass of free living microbes (Fig. 1A), thus reducing microbial competition for NO_3 (Fig. 1C), and, eventually also for amino compounds. Microbial biomass as determined within this study did comprise not only free living microorganisms but, despite roots being carefully removed from soil, also parts of the extramatrical mycelium of ectomycorrhizal fungal plant symbionts (Högberg and Högberg, 2002). However, the negative correlations between the abundance of *C. geophilum* as well as plant N uptake and total microbial biomass indicates that chloroform-labile microbial biomass as determined within this study is more representative for free living microorganisms than for mycorrhizal symbionts. Furthermore, in the present study, girdling did not affect the abundance of *C. geophilum* as the most abundant mycorrhizal fungal symbiont (Table 2), or the mycorrhizal colonization rate of fine roots in general (Pena et al., submitted for publication). Thus, the observed decrease in soil microbial biomass C and N appears to be mainly related to a decrease of free living microorganisms. These groups of free living microorganisms affected by girdling may have been sustained by the trees by the exudation of easily available C compounds into the rhizosphere. Zeller et al. (2008) reported lower microbial biomass in the first 3 months after girdling and later on similar values between control and girdling treatments in a spruce forest. This was attributed to the initial removal of carbohydrates from root exudation and subsequent C supply from dieback of roots. In contrast to our study, Högberg and Högberg (2002) found a decrease of soil microbial biomass of 32% following girdling in a boreal forest which was attributed to a mycorrhizal decline.

Since decreasing C availability negatively affects microbial activity in general and thus increases the amount of oxygen available, it should also decrease denitrification (Philippot et al., 2007). As alternative electron acceptors, many bacteria are able to use NO_3, which in turn is reduced stepwise to N_2. Therefore, not surprisingly, the number of denitrifiers tended to be lower at girdled plots, as the amount and quality of the C available in the plots with girdled trees was significantly reduced compared to the controls. However, most denitrifiers are facultative microbes and can switch their metabolism according to the conditions at their particular microsite. Therefore, a more dramatic reduction of denitrifiers was not observed.

Dannenmann et al. (2006) reported that a decrease in soil C availability in the Ah horizon of the investigated soil triggered by forest thinning led to a decrease in microbial immobilization of inorganic N in favour of gross nitrification, and thus, to increased soil NO_3 concentrations. Similarly, in the present study, increased NO_3 availability for beech trees at the girdled plots (Fig. 1D) was caused by a shift in the balance of microbial NO_3 production and consumption (increasing or stable gross nitrification rates, reduced nitrate immobilization rates; Fig. 1B,C) leading to a higher mean residence time of NO_3 in soil. Thus, girdling affected the balance of microbial nitrate production and consumption. Since tree NO_3 uptake at girdled plots compared to control plots was significantly higher in 2006, trees did not directly contribute to increased soil NO_3 concentrations, but may have profited from the higher NO_3 supply. However, it is likely that the shift in the balance of microbial NO_3 production and consumption was triggered by reduced rhizodeposition of carbohydrates in the roots, and thus, indirectly mediated by the trees.

4.4. Can soil C availability also explain the varying plant–microbe competition patterns between the sampling dates?

Independent from girdling effects, the sampling dates in two consecutive years were characterized by completely different N pools, microbial activity and plant N uptake rates, indicating different plant–microbe competition patterns (Fig. 1). In 2006, microbial biomass pools were half the size compared to 2007 (Fig. 1A). Furthermore, both gross rates of microbial N turnover and inorganic N pools were generally smaller in 2006 than in 2007 (Fig. 1B,C,D). In contrast, rates of plant N acquisition were in general higher in 2006 than in 2007. The severe disruption of microbial activity in 2006 may have been caused by drought periods and strong drying/rewetting cycles (Borken and Matzner, 2009) in the pre-sampling period. A severe breakdown of microbial activity by complete drying of the soil was confirmed for soil of the Tuttlingen site by Blagodatskaya et al. (in press). These authors showed that significantly more N_2O was produced after rewetting soil that was pre-incubated at low soil moisture compared to complete dry soil, thus supporting the idea of microbial community breakdown under severe drought conditions.

A drought- and drying/rewetting-induced breakdown of the free living microbial community in 2006 may explain that the DOC and DON pools were 2–2.5 times larger in 2006 than in 2007 (Fig. 1G). This might be related to (1) impaired degradation of DOM due to the disrupted microbial community, and (2) direct origin of DOM from the breakdown of microbial biomass. A very tight ratio between DOC and DON in 2006 (Table 2) supports the hypothesis of a predominantly microbial origin of DOM in 2006, and, furthermore, indicates that preferentially bacteria were negatively affected by the environmental conditions in 2006. The latter hypothesis is supported by the observation that the microbial C:N ratio was significantly higher in 2006 than in 2007 (Table 2) while total microbial biomass was significantly lower in 2006 than in 2007 (Fig. 1A). In contrast to microbial pools and fluxes, plant uptake as well as the abundance of *C. geophilum* was generally higher in 2006

Table 2
Mean C:N ratios of DOM and microbial biomass and the abundance of *Cenococcum geophilum*. Capital letters indicate significant differences between the sampling dates (years 2006 and 2007). The abundance of *C. geophilum* was calculated as number of root tips colonized with *C. geophilum* relative to the total number of root tips colonized with Ectomycorrhizae. Given are means of $n = 3$ plots (±SE), except for the abundance of *C. geophilum* at the girdling treatment in 2006, where only one pooled sample was analysed. There were no significant girdling treatment effects. C, control treatments; G, girdling treatments; 06, 07, year of sampling date.

	C06	G06	C07	G07
DOC/DON ratio	6.8 ± 0.9A	5.5 ± 0.5A	10.0 ± 1.9B	7.7 ± 0.8B
MBC/MBN ratio	11.5 ± 0.1A	11.7 ± 1.8A	9.6 ± 0.5B	9.4 ± 0.2B
C. geophilum	46.5 ± 4.1	45.4	35.2 ± 1.7	38.0 ± 5.0

than in 2007 (Fig. 1, Table 2). This indicates that plants and associated mycorrhizal fungal partners were less negatively affected by the meteorological conditions in the pre-sampling period or may have recovered faster, thus profiting from both high DON availability and reduced competition by free living microorganisms. Pena et al. (submitted for publication) showed that the ectomycorrhizal colonisation of vital root tips was unaffected between the sampling dates. This finding further supports the view that free living microorganisms were more negatively affected by the environmental conditions in 2006 than the plant–mycorrhizal symbionts.

The very tight C:N ratio of K_2SO_4-extractable DOM in 2006 (Table 2) implies that available C is limiting N turnover processes. Strong C limitation is also indicated by the pattern of microbial N turnover processes observed in 2006 (low gross ammonification, but gross nitrification of similar magnitude, while NH_4^+ immobilization was not significant; Fig. 1B,C). Under these conditions, mycorrhizal fungal symbionts should have higher competitive strength at the expense of free living microorganisms due to C supply by plants. Several studies suggested that C supply to microorganisms can claim a major role in the regulation of plant–microbe competition for N since the stimulation of microbial activity due to addition of labile C to the soil decreased plant N uptake and plant growth (Jonasson et al., 1996; Rutherford and Juma, 1992; Schmidt et al., 1997; Dunn et al., 2006). In the present study, C limitation might have been intensified by reduced rhizodeposition of carbohydrates under drought conditions (Borken and Matzner, 2009; Högberg and Read, 2006). These observations suggest that, similar to the observed girdling effects, beech may diminish the activity of free living microorganisms under drought stress by reduced C rhizodeposition, while C allocation to the most important mycorrhizal fungal symbiont *C. geophilum* persists at a sufficient rate. Thus, beech may strengthen its own competitive power under drought stress.

5. Conclusions

The present study widens our knowledge on the range of ecosystems where uptake of intact amino compounds contributes to tree nutrition to typical mountainous beech forests of central Europe. Actual belowground N fluxes in the investigated ecosystem appear to be closely mediated by strong competition for N between beech and free living microorganisms. By shifting the competitive balance for N acquisition in favour of plants and at the expense of free living microorganisms, girdling seemed to favour both increased NO_3 uptake and increased amino acid uptake by trees. A .similar effect on patterns of N partitioning between trees and microbes appeared to be mediated by intensive drought and drying/rewetting events, which led to a breakdown of the microbial community and severely impaired microbial competition for N in favour of N uptake by plants and its fungal symbiont *C. geophilum*. Competitive patterns of N partitioning between plants and microbes in the investigated ecosystem appeared to be closely linked to C availability and the associated dynamics of microbial biomass in soil. In turn, beech may regulate C availability to such an extent that microbial competition for N is impaired in order to favour its own competitive strength in N acquisition.

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Appendix 2

Carbon and nitrogen balance in beech roots under competitive pressure of soil-borne microorganisms induced by girdling, drought and glucose application.

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Carbon and nitrogen balance in beech roots under competitive pressure of soil-borne microorganisms induced by girdling, drought and glucose application

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Abstract. The goal of this work was to increase the understanding of factors regulating nitrogen (N) competition between roots and soil microbes. For this purpose, root assimilate supply was diminished or abolished in beech (*Fagus sylvatica* L.) seedlings by girdling, drought stress or a combination of both factors. This was revealed by ^{13}C tracer abundance in root tips after $^{13}CO_2$ pulse labelling of the shoots. Analysis of different root tip fractions revealed that only 6% were ectomycorrhizal. Carbon (C) allocation to ectomycorrhizal and vital non-mycorrhizal root tips was ~26% higher than to distorted root tips. Drought resulted in ~30% increased ammonium (NH_4^+) and amino acid concentrations in roots and ~65% increased soil NH_4^+ concentrations, probably because of lower consumption of NH_4^+ by free-living microorganisms. Root uptake of glutamine of 13 nmol g^{-1} fresh mass h^{-1} decreased 2-fold with drought, although the number of vital root tips did not decrease. Carbon content in biomass of free-living microbes increased with glucose application regardless of drought, resulting in significant depletion in soil nitrate (NO_3^-), root NH_4^+ and amino acid concentrations. Our results suggest that the root soil system of young beech trees was C-limited, and this prevented amino acid metabolism in roots and microbial NO_3^- consumption in the soil, thereby exerting feedback inhibition on uptake of inorganic N by roots. We suggest that rhizodeposition is a key link in regulating the plant–microbial N balance.

Additional keywords: competition, ectomycorrhiza, *Fagus sylvatica*, microorganisms, root demography, stable isotopes.

Introduction

Free-living microorganisms compete with plants for soil nitrogen (N) sources such as nitrate (NO_3^-), ammonium (NH_4^+) and organic N; e.g. in the form of free amino acids (Jackson *et al.* 2008). In undisturbed forest ecosystems, plant growth is usually N-limited, but the N concentration in soil generally increases after disturbance (Vitousek *et al.* 1979). Soil microbes play decisive roles in ecosystem processes related to nutrient cycling (van der Heijden *et al.* 2008). They transform organic N to NH_4^+ and further to NO_3^- by ammonification and nitrification, respectively. It is now well recognised that plants use not only inorganic forms of N, but also show significant uptake rates for certain amino acids (Lipson *et al.* 1999; Wallenda and Read 1999; Dannenmann *et al.* 2009; Näsholm *et al.* 2009). Since roots of temperate and boreal forest trees are colonised by ectomycorrhizal (EM) fungi that can also use organic and inorganic N sources (Wallander *et al.* 1997), EM fungi, plants and free-living microbes compete for all forms of N. The concentrations of NH_4^+ and NO_3^- in soil are, thus, dependent on the balance of microbial inorganic N production and immobilisation as well as on plant respective EM fungal N uptake (Booth *et al.* 2005; Dannenmann *et al.* 2006, 2009; Lindahl *et al.* 2007). However, the key processes influencing this balance are not well understood.

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Free soil microorganisms as well as EM fungi strongly depend on plants for their carbon (C) supply (Högberg *et al.* 2001; Heinemeyer *et al.* 2007). $\delta^{13}C$ analyses have shown that mycorrhizal hyphae are the main pathway for the release of plant-derived C into the soil (Godbold *et al.* 2006). When belowground C supply was inhibited by girdling, soil respiration decreased by ~40–65%, which was ascribed to the 'heterotrophic component' of soil CO_2 flux due to free microbes (Högberg *et al.* 2001; Andersen *et al.* 2005). Hyphal respiration of ectomycorrhizas contributed about ~25% of soil CO_2 flux (Heinemeyer *et al.* 2007). The inhibition of the respiratory activity of free soil microbes in girdling treatments can be released by glucose addition (Göttlicher *et al.* 2006). This indicates that changes in the availability of carbohydrates affect the activity of soil microbes thus influencing N mineralisation and immobilisation and further suggests that plant-derived carbohydrates may result in feedback regulation of root N metabolism.

Furthermore, the activity of soil microbes is sensitive to changes in water availability (Borken and Matzner 2008). Two factors may contribute to this sensitivity: the decrease in water as solvent and decreased C availability because of decreased synthesis and allocation of photoassimilates by the vegetation. Drought stress is, therefore, also expected to influence belowground N cycling.

The present study was conducted to increase our understanding of factors regulating the competition for N between trees and microorganisms. Towards this aim a controlled experiment was performed with young beech trees (*Fagus sylvatica* L.). Beech was chosen because it is the potentially dominant tree species in most Central European forests and was found to be susceptible to drought (Rennenberg *et al.* 2006). Beech seedlings are used for reforestation, e.g. after wind-breaks and for transformation of mono-cultures into mixed forests (Bolte *et al.* 2007). Although roots of mature trees are usually completely colonised by EM fungi, the degree of EM colonisation of planted seedlings is initially low (5–10%, Zeleznik *et al.* 2007). It is unknown how seedlings in this critical phase of establishment compete with soil microbes for nutrients. In the present study, C supply to microbes was modulated by girdling and drought to suppress the transfer of recent photosynthate to the soil and by glucose application, a common practice in soil science to boost microbial growth. We hypothetised that (i) reduced belowground C transfer leads to suppressed microbial N immobilisation and increased soil N concentrations; this will, in turn, increase root N supply and (ii) increased belowground C availability increases the competetiveness of free soil microbes; this will decrease soil N concentrations and thereby, limit root N uptake.

Materials and methods

Plant cultivation

One-year-old beech plants (*Fagus sylvatica* L. provenance: Schwäbische Alb, Germany) were obtained from a tree nursery (Schlegel and Co., Riedlingen, Germany) and planted in November 2006 into containers (eight plants per container, 80 containers). The containers (Ecco4-Stapelboxen, Walther Faltsysteme, Kevelaer, Germany) had dimensions of $355 \times 255 \times 315$ mm and were filled with ~25 L soil. The bottom was perforated (two holes) and covered with a 20 mm thick layer of foamed clay on which a fleece mat (17 g m^{-2}) was placed. The container was filled with soil obtained near the edge of a forest close to the research station Tuttlingen (8°45′E, 47°59′N) (see Dannenmann *et al.* 2009 for more detail). Since the soil was from a clearing it represents a typical soil for reforestation and was, therefore, used for this study. The soil was loamy clay classified as Rendzic Leptosol (Sceletic) consisting of 71% clay, 24% silt and 5% sand. The pH was 7.4 (measured in 0.01 M $CaCl_2$).

Soil horizons were collected to a depth of 1 m, mixed and homogenised by sieving (16 mm). The plants overwintered outdoors under ambient conditions. The containers were transferred into air-conditioned greenhouse cabinets on 29 March 2007 and plants were cultivated under conditions mimicking outside temperature and RH until harvest in the first week of August, with mean day/night air temperatures of 22.6/15.0°C and relative day/night RH of 56/73%. Photosynthetic active radiation fluctuated from 91 to 674 µmol quanta $m^{-2} s^{-1}$, corresponding to a mean daily dose of 8186 mmol m^{-2} day^{-1}. The mean daily maximum photosynthetic radiation was 424 µmol quanta $m^{-2} s^{-1}$. From 1 April to 30 May 2007, all plants were watered regularly with demineralised water (total amount 22.18 L per container during this period of time corresponding to 80.1 L m^{-2}). The total water supply was similar to the precipitation at the reference field site Tuttlingen (Germany) of 57.6 L m^{-2} from April to July.

Experimental treatments

The experimental treatments were conducted as fully randomised block design with three factors (controls, girdled trees and glucose application) and two water levels. For each treatment, eight containers were used. Drought treatment was started on 1 June and continued for 9 weeks. Drought-exposed plants received 4.97 L and well irrigated plants a total amount of 13.65 L water during this time, corresponding to 57.7 and 156 L m^{-2}, respectively. The irrigation regime was based on precipitation at the reference site Tuttlingen using the monthly mean precipitation between May and September of the years 2001–04 of 87 L m^{-2} as target value. Drought stressed plants received ~1/3 of this value, i.e. 33 L m^{-2}.

One third of the containers were irrigated with glucose solution (100 g in 500 mL demineralised water). The first application was on 30 May and afterwards in intervals of 15 days (five applications in total). Although the osmotic potential of the glucose solutions was low (–2.7 MPa), the 5-to 10-fold dilution of this solution in the containers, which contained soil water volumes of ~5 L (controls) and 2.5 L (stressed), respectively, was not expected to generate additional osmotic stress. Non-glucose treated plants received the same amount of water. Girdling took place on 11 and 12 of June. A 10 mm wide bark strip was removed at the bottom ~5 cm above ground around the whole stem circumference.

One set of plants of each except the glucose treatments was subjected to a $^{13}CO_2$ label pulse for 4 h (0845 to 1245 hours) on 24 July. For this purpose, the plants were enclosed in a tunnel of

plastic foil (ethylene-tetrafluorethylene, thickness 80 µm) sized 1.20 × 5.04 × 1.30 m. Air that was depleted in CO_2 by soda lime to 280 µmol mol^{-1} and afterwards ^{13}C enriched CO_2 (2% CO_2 with ≥95% ^{13}C in 98% N_2; Spectra Gases Ltd, Littleport, UK) was added to the tunnel atmosphere at a flow rate of ~1 L min^{-1}. The $^{12}CO_2$ concentration in the tunnel was controlled photoacustically with a CO_2 controller (Siemens, Typ 7MB1300–0AA10, Ditzingen, Germany) and maintained at an average concentration of 380 µmol mol^{-1}. The absolute CO_2 concentration during labelling was raised from 300 to 800 µmol mol^{-1} with an average of 668 µmol mol^{-1} and mean ^{13}C abundance of 66 atom-% (±2 atom-% s.e.). Twelve days after pulse labelling, harvest started and it took 3 days for the plant and soil samples to be collected.

Ecophysiological measurements

Photosynthesis and transpiration were measured at ambient CO_2 concentration, ambient temperature and saturating light intensity (400 µmol m^{-2} s^{-1} PPFD) repeatedly during cultivation of the plants in the greenhouse using a portable gas-exchange measuring system (HCM-1000, H. Waltz GmbH, Effeltrich, Germany).

Soil sampling and analysis

Soil samples were taken after roots had been removed (see below) and stored at 4°C in polyethylene bags in darkness until analysis. NO_3^- and NH_4^+ concentrations were measured by extracting soil with 0.5 M K_2SO_4 and subsequent colourimetric determination by continuous flow analysis (Skalar Analytik GmbH, Erkelenz, Germany) in a commercial laboratory (Laboratory Dr. Janssen, Gillersheim, Germany) as described by Dannenmann *et al.* (2006). Gravimetric soil water content was determined by drying at 105°C for 24 h. Total C and N contents of the soil were measured by dry combustion (Elementar, vario EL CN Analyzer, Hanau, Germany). Inorganic C concentrations of samples were determined after combustion of samples for 3 h at 550°C in a muffle oven. The organic C concentrations of the samples were calculated as the difference between total and inorganic C.

Microbial biomass and gross rates of microbial N mineralisation

Gross rates of N mineralisation (ammonification) were analysed by use of the ^{15}N pool dilution technique as described previously (Dannenmann *et al.* 2006, 2009) with the modification that 0.5 M K_2SO_4 was used as soil extractant instead of 1 M KCl. Microbial biomass was determined by the chloroform-fumigation extraction method as described previously (Dannenmann *et al.* 2006).

Plant harvest

At harvest the biomass of each tree was determined after separating foliage, aboveground wood tissues and roots. Soil particles adherent to the roots were removed by careful washing. Biomass of detached roots was attributed proportionally to the individual trees. Roots of two trees grown in the centre were further separated into fine roots (<1 mm in diameter) and coarse roots (>1 mm in diameter).

Aliquots of fresh material of the different fractions were taken for subsequent morphological, biochemical and mycorrhizal analysis. The remaining biomass fractions were dried at 65°C to constant mass.

Fine root materials for determination of total N and N-containing metabolites (total amino acids, soluble protein, NO_3^- and NH_4^+) were immediately frozen in liquid nitrogen after harvest. Samples were stored at −80°C until further analysis.

Mycorrhizal analysis and ^{13}C analysis in root tips

For mycorrhizal analysis root samples of two plants grown in the centre of each container were used. These roots were considered as one replicate and amounted to 0.24 g per sample. A total of eight replicates per treatment were analysed for mycorrhizal colonisation. Roots were spread under a compound microscope (Stemi SV 11, Zeiss, Jena, Germany). Morphotyping was conducted as described elsewhere (Agerer 1987–2006; Allen *et al.* 1995). Vital non-mycorrhizal, mycorrhizal and brown root tips, as well as shrunken non-mycorrhizal, shrunken mycorrhizal and black distorted root tips were cut under the compound microscope and pooled according to root tip class and treatment. The root tips were used for $\delta^{13}C$ analysis (see below) and for internal transcribed spacer (ITS) sequencing as described previously (Druebert *et al.* 2009) and sequences were deposited at NCBI databank under FJ997646 for *Hebeloma sachariolens* and FJ997647 for *Tuber* sp.

For analysis of total C and $\delta^{13}C$ in different root tip fractions, samples were freeze-dried for 24 h. Subsamples of 0.15–0.49 mg were weighed with a super-micro balance (S4, Sartorius, Göttingen, Germany) in 5 × 9 mm tin capsules (Hekatech, Wegberg, Germany) and the $\delta^{13}C$ isotope ratios were determined at the Centre for Stable Isotope Research and Analysis (University Göttingen) with an isotope ratio mass spectrometer (IRMS Delta Plus, Thermo Finnigan Mat, Bremen, Germany) coupled to an elemental analyzer (EuroVektor, HEKAtech, Wegberg, Germany). Isotope ratios are expressed in delta notation (in ‰) relative to the Vienna Pee Dee Belemnite and used to calculate ^{13}C atom-% as $^{13}C/(^{13}C + ^{12}C) \times 100$.

Determination of N uptake by fine roots

Net N uptake in fine roots of beech seedlings was examined before harvests using the ^{15}N enrichment technique as described by Gessler *et al.* (1998*a*). For this purpose, one side of each box was cut open and roots were carefully dug out of the soil, washed with distilled water and dried with paper towel. The roots still attached to the trees were incubated for 2 h (starting from 1000 until 1200 hours) in 4 mL of an artificial soil solution of similar pH and concentrations of inorganic anions and cations as in the soil water obtained from lysimeters at the Tuttlingen experimental beech forest (Gessler *et al.* 2005). The concentrations of N-containing compounds were 10 µM NO_3^- (as KNO_3), 0.1 µM NH_4^+ (as NH_4Cl) and 2.5 µM of glutamine. The artificial solutions contained ^{15}N-label either as ^{15}N-nitrate, ^{15}N-ammonium or $^{15}N/^{13}C$-double labelled glutamine. In addition, control solutions without ^{15}N-label were used to account for the natural abundance of ^{15}N in the roots.

After 2 h of incubation, the submerged parts of the roots and a small (~8–10 mm), moistened part outside the solution were cut off, washed twice in distilled water, dried with paper towels and stored in liquid nitrogen until further analysis. The calculation of net uptake rates (nmol g^{-1} FW h^{-1}) was based on the incorporation of ^{15}N into root fresh mass. Based on ^{13}C incorporated into root fresh mass, glutamine uptake rates were generally significantly lower, indicating degradation of glutamine during the 2-h incubation time in the incubation solution or on the root surface at the relatively low concentrations applied or potential further metabolisation of C after uptake of intact amino acid molecules including ^{13}C respiration losses.

Total N concentration and $\delta^{15}N$ ratio of oven-dried (48 h, 60°C) root material (0.5–1.0 mg) were determined using an EA-IRMS as above. Isotope ratios are expressed in delta notation (in ‰), relative to the Vienna Pee Dee Belemnite (C) and air standard (N) or as atom-%.

Soluble protein and amino acids in fine roots

Frozen root material was ground in liquid nitrogen with mortar and pestle to a fine powder and kept at −80°C until further analysis. For soluble protein analysis, ~0.05 g of frozen root tissue were extracted in 1 mL buffer (50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 15% glycerol (v : v), phenylmethylsulfonylchloride (PMSF, 1 mM), dithiothreitol (DTT, 5 mM) and 0.1% Triton X-100) by mixing and incubation for 30 min at 4°C. After centrifugation at 14 000*g* for 10 min at 4°C, protein contents in 50 µL aliquots of the supernatant were quantified by adding 1 mL of Bradford Reagent (Amresco Inc., Solon, Ohio, USA). After 10 min incubation at room temperature in the dark, the optical density was measured in a UV-DU650 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA) at 595 nm. Bovine serum albumin (BSA; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, A-6918) was used as the standard.

Extraction of free amino acids from ~0.5 mg frozen, ground fine roots was conducted according to the method by Winter *et al.* (1992). Total amino acid contents were quantified with ninhydrin reagent as described previously (Liu *et al.* 2005). Glutamine was used as the standard.

NH_4^+ and NO_3^- in fine roots

For the extraction of anions, 0.1 g washed polyvinylpyrrolidone was soaked with 1 mL distilled water for 2 h before adding 0.05 g of ground root material to the mixture. After shaking in the dark for 1 h at 4°C, the samples were incubated at 95°C for 10 min, combined with an autosampler (AS 3500, Thermo Separation 252 Products, Piscataway, NJ, USA) as described in detail by Dannenmann *et al.* (2009). An anion mixture of NO_3^-, PO_4^{3-}, SO_3^- and SO_4^{2-} and a cation mixture of NH_4^+, Na^+, K^+, Mg^{2+} and Ca^{2+} in distilled water were used as standards. Data were analysed using the PeakNet software package (V. 4.3, Dionex, Idstein, Germany).

Statistical analysis

Statistical analysis was performed using Statgraphics Plus 3.0 (StatPoint, Inc., St Louis, MO, USA). Data in tables and figures are means (±s.e.) when not indicated otherwise. The number of replicates varied because it was not possible to analyse all plants in all containers and is indicated in the table and figure legends. Data were subjected to ANOVA or multiple analyses of variance and subsequently to a multiple range test. Differences between means were considered significant at $P \leq 0.05$.

Results

Influence of drought, girdling and glucose application on soil water content and plant performance

Diminished irrigation for about 2 months resulted in significant reduction of the soil water content compared with control conditions where water was supplied according to the precipitation of a reference field site in southern Germany, Tuttlingen (Table 1). Plant girdling or addition of glucose to the irrigation water had no influence on the relative soil water contents (Table 1). The concentrations of organic C (30.3 ± 1.0 mg g^{-1} soil dry mass) or the total soil N (2.79 ± 0.07 mg g^{-1} soil dry mass) were not affected by any of the treatments. Plants grown with limited water supply were

Table 1. Relative soil water content (SWC), whole-plant biomass, net photosynthesis (*A*), stomatal conductance (g H_2O) and water use efficiency (WUE) of young beech (*Fagus sylvatica*) trees

Drought stress, glucose applications and girdling were applied as described in 'Materials and methods'. Plants were harvested after about 2 months of drought stress (DS). *P*-values and interactions were calculated by multiple analyses of variance using treatments (control, girdle, glucose) and DS as variables. Data indicate means of three replicates (SWC) and eight or more replicates other parameters (±s.e.)

Treatment	SWC (%)	Biomass (g DW $plant^{-1}$)	*A* (µmol m^{-2} s^{-1})	g (H_2O) (mmol m^{-2} s^{-1})	WUE (µmol $mmol^{-1}$)
Control	20.9 ± 0.7	10.5 ± 0.5	5.7 ± 0.4	98.4 ± 8.2	2.7 ± 0.1
Control + DS	11.4 ± 0.9	9.5 ± 0.7	1.6 ± 0.3	16.5 ± 3.1	3.5 ± 0.2
Girdle	26.8 ± 4.5	9.6 ± 0.5	2.6 ± 0.3	56.1 ± 5.1	1.9 ± 0.1
Girdle + DS	12.3 ± 1.4	9.2 ± 0.4	0.6 ± 0.1	14.1 ± 1.0	1.4 ± 0.2
Glucose	24.7 ± 2.8	11.3 ± 0.6	6.5 ± 0.4	108.6 ± 4.3	3.0 ± 0.3
Glucose + DS	13.9 ± 0.4	9.7 ± 0.5	1.3 ± 0.2	18.2 ± 4.9	3.4 ± 0.9
P-value (Treatment)	0.290	0.142	0.000	0.000	0.317
P-value (DS)	0.000	0.024	0.000	0.000	0.000
Interaction (Treat × DS)	0.543	0.516	0.000	0.000	0.005

moderately drought stressed. Their pre-dawn water potential dropped to -1.30 ± 0.22 MPa, which reduced their hydraulic conductivity by ~20% (Cochard *et al.* 1999; Lemoine *et al.* 2002), whereas that of well irrigated plants was at -0.16 ± 0.02 MPa ($P \leq 0.001$).

Beech has a determined growth pattern; forming only one shoot with new leaves in spring. Since drought treatment started after full expansion of the annual flush, whole-plant biomass was only slightly decreased in response to drought (Table 1), even though significant effects were observed with respect to root biomass (see below). Girdling or glucose application to the soil had no influence on plant biomass, although effects on photosynthetic CO_2 assimilation and stomatal conductance were observed (Table 1). The water use efficiency of girdled plants was lower than that of non-girdled plants. Glucose application had no effect on water use efficiency (Table 1).

Influence of drought, girdling and glucose application on gross rates of N mineralisation and microbial biomass C and N

The concentrations of NH_4^+ in soil were generally low, decreased in response to glucose treatment and increased in response to drought (Table 2). In contrast to NH_4^+, the concentrations of NO_3^- were high (Table 2). The NO_3^- concentrations decreased substantially in response to glucose application (Table 2). Small but significant NO_3^- increases were observed in response to drought but only in control and girdling treatments (significant interaction, Table 2).

Gross rates of N mineralisation were higher in the soil below girdled plants than after glucose application (Table 2). Microbial biomass C generally tended to be lower under drought conditions; however, this effect was not significant (Fig. 1*a*). Glucose addition significantly increased microbial biomass (Fig. 1*a*). Drought treatment reduced microbial biomass N for all treatments (Fig. 1*b*, significant for the girdling and glucose treatment). Apparently, microbial activity in the soil of controls was C limited. This limitation may have contributed to the high soil NO_3^- concentrations in the absence of glucose addition (Table 2).

Influence of drought, girdling and glucose application on root and mycorrhizal performance

Drought caused an ~18% decrease in the root water content, which was apparent from the significant increase in the dry-to-fresh mass ratio of roots from drought stressed trees (Table 3). Root biomass was significantly decreased in response to drought and girdling (Table 3). These losses also affected the root-to-shoot ratio of the plants (Table 3).

We distinguished vital and distorted root tips and found no significant influence of girdling or glucose treatment on the numbers of vital root tips per unit of biomass (Table 3). However, in combination with drought, the number of shrunken root tips increased significantly for the girdled trees (Table 3). Since root degradation has turnover times of 1 year or more and degradation of ectomycorrhizal roots is even slower (Matamala *et al.* 2003; Langley *et al.* 2006), decomposition is too slow to result in differences in the fractions of vital and dead root tips in the present study. Therefore, we conclude that girdled trees exposed to drought stress, had an increased formation and mortality of roots compared with non-girdled trees.

The colonisation with mycorrhizal fungi was low amounting to 3.4 ± 1.7% of all root tips and to 6.6 ± 3.3% of living root tips (Table 4). This was probably caused by relatively high concentrations of inorganic N in the soil (Table 2), typical of disturbed soils and because young nursery-grown plants were used whose EM colonisation was low (<5% before planting into the growth boxes). Morphotyping and ITS sequencing revealed only two EM species, *Hebeloma sacchariolens* and *Tuber* sp., respectively (Fig. S1 available as an Accessory Publication to this paper). *Tuber* sp. was more abundant (mean 2.9 ± 1.6%) than *H. sacchariolens* (mean 0.4 ± 0.3%) and formed a thick mantle around the root tip (Fig. S1). Furthermore, brown and black root tips were observed resembling mycorrhizal root tips (http://www.deemy.de/; *Tomentella ferruginea*, *Fagirhiza fusca*, *Fagirhiza spinulosa*, *Fagirhiza setifera*). However, ITS sequencing gave no results and cross-sections did not reveal typical mycorrhizal structures but indicated different degrees of vitality of brown and black tip forms (Fig. S1).

Table 2. Inorganic N concentrations and gross rates of N mineralisation (ammonification) by microorganisms in soil of containers planted with young beech (*Fagus sylvatica*) trees

Drought stress (DS), glucose applications and girdling were applied as described in 'Materials and methods'. *P*-values and interactions were calculated by multiple analyses of variance using treatments (control, girdle, glucose) and DS as variables. Data indicate means of nine replicates (NH_4^+, NO_3^-) or three replicates of gross rates of N mineralisation (±s.e.). SDM, Soil dry mass

Treatment	Soil NH_4^+ (µg g^{-1} SDM)	Soil NO_3^- (µg g^{-1} SDM)	Gross N mineralisation (mg N kg^{-1} SDM day^{-1})
Control	0.8 ± 0.1	21.1 ± 0.5	1.7 ± 0.2
Control + DS	1.3 ± 0.1	25.1 ± 0.4	1.7 ± 0.3
Girdle	0.8 ± 0.0	33.9 ± 2.4	2.1 ± 0.2
Girdle + DS	1.3 ± 0.1	28.4 ± 1.4	2.4 ± 0.6
Glucose	0.6 ± 0.0	0.3 ± 0.1	0.7 ± 0.0
Glucose + DS	0.9 ± 0.2	0.4 ± 0.1	1.6 ± 0.2
P-value (Treatment)	0.003	0.000	0.021
P-value (DS)	0.000	0.641	0.155
Interaction (Treatment × DS)	0.599	0.001	0.361

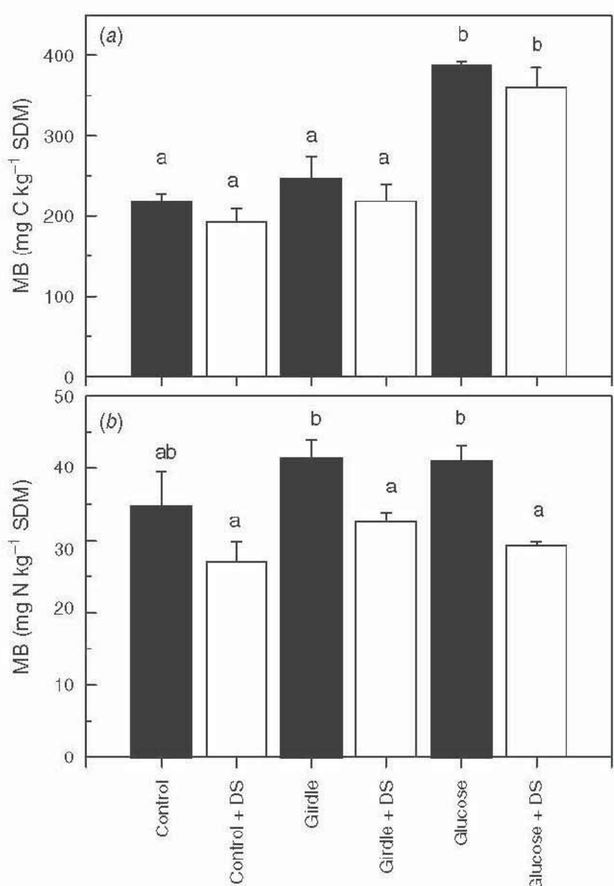

Fig. 1. Soil microbial biomass (MB) determined as (*a*) chloroform-labile carbon and (*b*) nitrogen in soil of containers planted with young beech (*Fagus sylvatica*) trees. Drought stress (DS, open bars), glucose applications and girdling were applied as described in the Materials and methods section. Data indicate means of nine replicates (±s.e.). SDM, soil dry mass. Different letters indicate significant differences with $P \leq 0.05$.

Vital brown and distorted black root tips were most abundant, amounting to 34.5 ± 5.1% and 26.9 ± 6.4% of all root tips, respectively. In addition to these structures, shrunken non-mycorrhizal root tips, shrunken mycorrhizal and vital non-mycorrhizal tips were found.

Vital fractions of non-mycorrhizal and mycorrhizal root tips were not affected by any of the treatments (Table 4). In contrast, the fraction of black root tips decreased and dead mycorrhizal root tips increased significantly after drought stress (Table 4).

Influence of drought, girdling and glucose application on C allocation to roots

Total C concentration was 8% lower in drought-stressed roots than in roots of well watered plants, whereas girdling or glucose application had no influence (see Table S1 available as an Accessory Publication to this paper). To assess belowground C allocation to and C accumulation in vital and distorted root tips under drought and girdling, plants were pulse-labelled for 4 h by exposure to $^{13}CO_2$ and vital and distorted root tips were harvested after 12 days for ^{13}C analysis (Fig. 2). Significant ^{13}C enrichment was present in vital root tips of control plants, whereas drought and girdling suppressed, but did not eliminate, ^{13}C enrichment (Fig. 2). Only girdling in combination with drought abolished belowground C accumulation in fine roots completely because the ^{13}C value in the root fraction of these plants was as low as in non-labelled plants (Fig. 2). A comparison between vital and distorted roots tips showed a significant reduction in ^{13}C incorporation in the latter fraction across all treatments ($P = 0.044$). However, distorted root tips from control plants accumulated ^{13}C well above the threshold of non-labelled plants, suggesting that these roots tips might still have had low physiological activity or that this reflected a portion of root tips that died after labelling. Under all other conditions the ^{13}C signature in the fraction of distorted root tips was not significantly different from the background.

Influence of drought, girdling and glucose application on root N metabolism

To assess the consequences of the different treatments, which resulted in changes of C flux to the roots and N availability in the soil, on root physiology, the concentrations of total N, soluble protein, NH_4^+, NO_3^- and amino acids as well as the uptake rates of inorganic and organic N were determined. Soluble protein

Table 3. Ratio of dry-to-fresh weight, whole-plant root biomass, root-to-shoot ratio and number of living or dead root tips (RT) of young beech (*Fagus sylvatica*) trees

Drought stress (DS), glucose applications and girdling were applied as described in 'Materials and methods'. *P*-values and interactions were calculated by multiple analyses of variance using treatments (control, girdle, glucose) and DS as variables. Data indicate means of eight replicates (±s.e.)

Treatment	DW/FW	Root biomass (g DM plant^{-1})	Root/shoot	Vital RT (# g^{-1} FW)	Shrunken RT (# g^{-1} FW)
Control	0.34 ± 0.08	5.30 ± 0.25	1.03 ± 0.04	2345 ± 455	1829 ± 274
Control + DS	0.46 ± 0.05	4.19 ± 0.22	0.82 ± 0.04	1572 ± 315	1243 ± 196
Girdle	0.31 ± 0.08	3.25 ± 0.15	0.52 ± 0.02	1576 ± 564	1961 ± 380
Girdle + DS	0.43 ± 0.06	2.95 ± 0.14	0.47 ± 0.01	1623 ± 270	3087 ± 466
Glucose	0.37 ± 0.07	5.63 ± 0.23	1.02 ± 0.03	1510 ± 195	1571 ± 222
Glucose + DS	0.48 ± 0.05	4.56 ± 0.21	0.89 ± 0.02	1296 ± 168	1755 ± 282
P-value (Treatment)	0.016	<0.001	<0.001	0.297	0.006
P-value (DS)	0.000	<0.001	<0.001	0.288	0.357
Interaction (Treatment × DS)	0.891	0.073	0.016	0.508	0.035

Table 4. Fractions of vital and distorted ectomycorrhizal (EM) and non-mycorrhizal (NM) root tips (RT) of young beech (*Fagus sylvatica*) trees
Brown and black roots tips were classified as vital and distorted according to Fig. 2. Drought stress (DS), glucose applications and girdling were applied as described in 'Materials and methods'. *P*-values and interactions were calculated by multiple analyses of variance using treatments (control, girdle, glucose) and DS as variables. Data indicate means of eight replicates (±s.e.). Sum of all root tips = 100%

	Fractions of vital root tips			Fractions of distorted root tips		
	EM RT (%)	Brown RT (%)	NM (%)	Dry EM (%)	Black RT (%)	Dry NM (%)
Control	5.3 ± 2.2	37.7 ± 4.5	11.1 ± 3.2	7.9 ± 3.8	29.0 ± 8.3	8.9 ± 3.6
Control + DS	3.5 ± 2.0	39.4 ± 4.6	10.6 ± 2.3	25.2 ± 5.6	13.6 ± 4.5	7.5 ± 2.2
Girdle	1.5 ± 0.8	32.7 ± 7.7	5.8 ± 2.9	22.3 ± 5.8	31.0 ± 9.9	6.6 ± 0.9
Girdle + DS	0.6 ± 0.4	28.0 ± 4.4	7.2 ± 2.4	32.7 ± 4.6	21.1 ± 5.5	10.5 ± 3.4
Glucose	1.0 ± 0.5	40.7 ± 4.0	8.1 ± 2.9	7.0 ± 1.1	40.6 ± 5.0	2.6 ± 0.6
Glucose + DS	8.3 ± 4.5	28.6 ± 5.2	7.4 ± 1.7	25.5 ± 5.0	26.1 ± 5.5	4.1 ± 0.9
P-value (Treatment)	0.210	0.301	0.240	0.029	0.211	0.052
P-value (DS)	0.418	0.247	0.975	0.000	0.020	0.485
Interaction (Treatment × DS)	0.098	0.428	0.910	0.641	0.908	0.537

contents (6.8 ± 0.4 mg g^{-1} DW) were unaffected by girdling or addition of glucose (*P* = 0.403) or drought stress (*P* = 0.928). The roots contained low concentrations of NO_3^-, which increased only in plants in which C accumulation in vital root tips was completely absent as a consequence of the combination of girdling and drought stress (Fig. 3*a*). NH_4^+ concentrations were high under all conditions and were particularly enriched in drought-stressed control and in drought-stressed and girdled plants (Fig. 3*b*). Glucose application resulted in significant decreases in NH_4^+ concentrations regardless of whether the plants were drought stressed or not (Fig. 3*b*). Free amino acid levels showed similar patterns as NH_4^+ in response to drought, girdling and glucose application (Fig. 3*c*).

The uptake of NH_4^+ was below the limit of detection of the method applied (0.44 nmol h^{-1} g^{-1} FW). The same was true for NO_3^- uptake, except for roots of plants grown in soil with glucose addition. These roots showed significant NO_3^- uptake rates, which dropped to about half when plants were drought stressed (Fig. 3*d*). Significant uptake rates were found for glutamine under all conditions tested (Fig. 3*f*). Drought stress resulted in about 2-fold reductions in glutamine uptake rates. Total N contents were increased in response to girdling from 1.06 to 1.13% and decreased to 0.88% in response to glucose exposure (Table S1). Drought caused slight decreases in total N contents (Table S1).

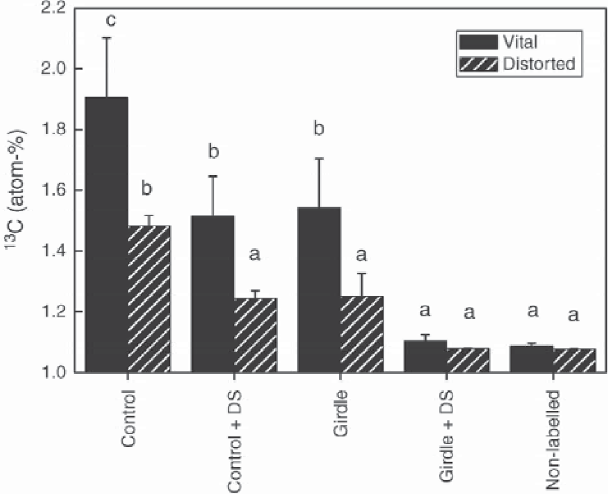

Fig. 2. ^{13}C atom-% in vital and distorted root tips after pulse labelling with $^{13}CO_2$. Drought stress (DS) and girdling were conducted as described in the Materials and methods section. Data indicate means of three replicates (±s.e.). Each replicate consists of ~200 pooled roots tips. Different letters indicate significant differences at $P \leq 0.05$.

Discussion

Drought and glucose application have contrasting effects on soil processes driven by free-living microbes

Combined studies of microbial soil processes and root physiology investigating the competition for N under changing C fluxes are scarce. Here, we have successfully manipulated the availability of inorganic N in the soil by glucose application and by modification of C supply to roots by girdling and drought. Via a shift in the balance of inorganic N production and consumption in favour of autotrophic gross nitrification and at the expense of heterotrophic NO_3^- immobilisation, C limitation may have facilitated high soil NO_3^- concentrations in the absence of glucose (Dannenmann *et al.* 2006) (Table 1). Conversely, glucose addition can be expected to have altered patterns of NH_4^+ partitioning to competing microbial processes by influencing the relative importance of autotrophic nitrification at 'both ends': heterotrophic microbial NH_4^+ immobilisation was promoted, thus, limiting substrate availability for the competitively inferior process of autotrophic nitrification. Furthermore, the consumption of NO_3^- via microbial immobilisation was promoted (Booth *et al.* 2005; Dannenmann *et al.* 2006).

Tree girdling significantly increased gross ammonification (Table 2). Zeller *et al.* (2008) found increased N mineralisation after tree girdling, which was linked to fine root mortality and turnover. The same may apply in the present study, where we found strong evidence that tree girdling increased the mortality of roots (Table 3). However, in contrast to field studies where girdling significantly decreased microbial biomass C and N in the soil (Göttlicher *et al.* 2006; Giesler *et al.* 2007; Dannenmann *et al.* 2009), a suppression in microbial biomass was not observed in our experiment. Mature trees probably release much higher amounts of C-containing compounds into the soil

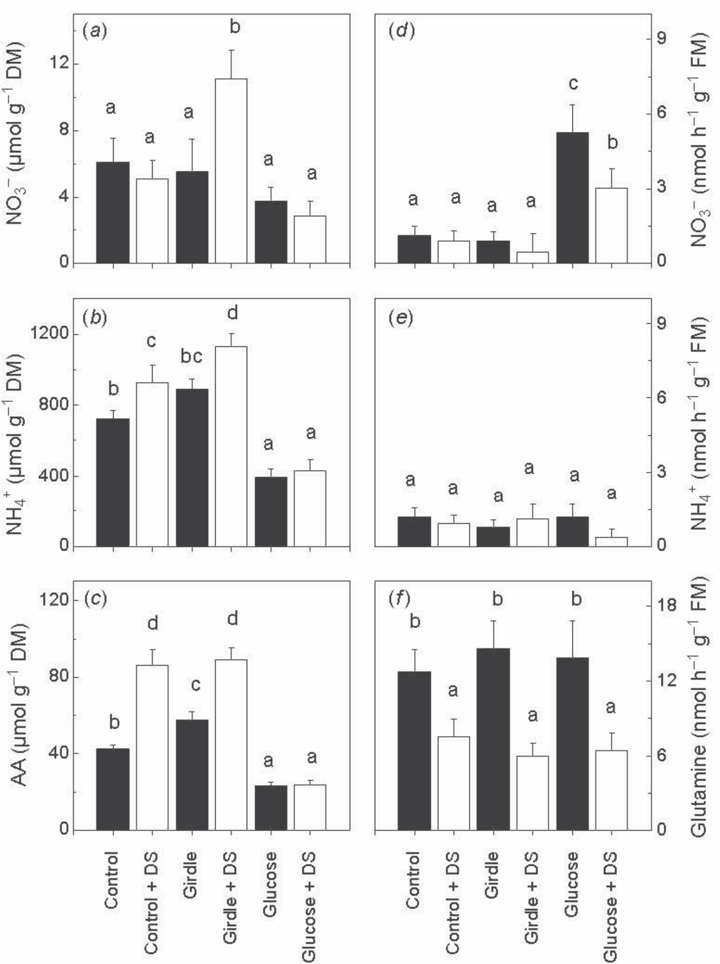

Fig. 3. (*a*) Nitrate, (*b*) ammonium and (*c*) free amino acid concentrations as well as uptake rates for (*d*) nitrate, (*e*) ammonium and (*f*) glutamine in roots of young beech (*Fagus sylvatica*) trees. Drought stress (DS), glucose applications and girdling were applied as described in the Materials and methods section. Data indicate means of 12–16 replicates (±s.e.). Different letters indicate significant differences at $P \leq 0.05$.

via rhizodeposition than the young saplings used here, so it can be expected that microbial biomass responds sensitive to changes in this supply route. Indeed, microbial biomass in beech forests was about one order of magnitude higher than that found here (Dannenmann *et al.* 2006, 2009). As glucose application increased N immobilisation in microbial biomass it is obvious that the free soil microflora was C limited. Therefore, we conclude that the competitive strength of soil microbes for N was increased by carbohydrate addition and that this resulted in diminished NO_3^- concentrations in the soil.

Drought, girdling and glucose affect root demography and mycorrhizas

In contrast to mature beech trees, whose root tips are usually 100% colonised (Rumberger *et al.* 2004; Buée *et al.* 2005), the young trees used in our study exhibited only low EM colonisation. Low colonisation rates have typically been found under high N supply (Wallenda and Kottke 1998) and on young and small seedlings (Zeleznik *et al.* 2007; Dučić *et al.* 2009). Furthermore, mycorrhizal colonisation is also strongly dependent on C supply to the roots (Druebert *et al.* 2009). In the present study, the combination of limited C supply for mycorrhizal colonisation and high availability of NO_3^- for plant nutrition may have prevented root colonisation by EM. Since mycorrhizal abundance was low it is unlikely that EM fungi contributed strongly to root N uptake processes. In contrast, in fully EM-colonised beech roots the abundance of certain fungi such as *Cenococcum geophilum* correlated with plant glutamine uptake (Dannenmann *et al.* 2009).

It was notable that the fraction of living root tips was unaffected by all treatments and that only complete interruption of C transport to the roots, which was achieved by girdling in combination with drought, resulted in significant increases in distorted root tips (Table 3). Since no incorporation of the ^{13}C label was found the increased root

turnover must have been fuelled by stored carbohydrates as observed for girdled beech trees under field conditions (Pena *et al.* 2010). Field studies in beech forests also revealed high root production rates under drought stress, which compensated higher root mortality under these conditions (Meier and Leuschner 2008). In contrast to our study, an increased root-to-shoot ratio was found for high light-exposed beech after drought stress (Löf *et al.* 2005). However, the increase was mainly caused by decreases in aboveground biomass and not by increases in root biomass. In our study, mild drought stress did not induce severe leaf shedding but diminished belowground C allocation. This might have caused a loss in root biomass resulting in a diminished root-to-shoot ratio.

We noted that the fraction of distorted root tips did not increase in response to drought as single factor despite significant decreases in soil water content (Table 1). The observation that low but significant C allocation was still possible under these conditions suggests that significant fluctuations in soil water content can be tolerated by beech, if C supply is warranted. Ruehr *et al.* (2009) showed that drought strongly delayed but did not abolish belowground C allocation in beech. ^{13}C enrichment in vital root tips after girdling indicates that the effect of this treatment on belowground C flux was similar to that of drought. Why girdling was unsuccessful in preventing C transfer to roots, remains unclear and contrasts previous studies (Druebert *et al.* 2009). Since beech stem wood contains not only radial but also axial parenchyma cells, partial bridging of the interrupted belowground C route by these tissues appears feasible.

Overall maintenance of the fraction of vital root tips under limited or interrupted photosynthate transfer suggests that the competition for N was not affected by limitations in fine root production.

Root N metabolism is linked with microbial-driven soil processes

Beech roots as well as mycorrhizal fungi can take up organic as well as inorganic N compounds (Gessler *et al.* 1998*a*; Wallenda and Read 1999; Chang and Matzner 2000; Templer and Dawson 2004; Dannenmann *et al.* 2009). Thus, plant and fungal components of N uptake are usually not distinguishable. Since typical mycorrhizal root tips were rare in our study, it is likely that N uptake, notably only in the form of glutamine, was mainly achieved by non-mycorrhizal roots. Drought stress decreased glutamine uptake, which might have been caused by lower physiological activity as a consequence of water loss or lower energy supply under these conditions.

At the first glance, lacking NO_3^- or NH_4^+ uptake was surprising and contrasts field observations (Gessler *et al.* 1998*b*; Chang and Matzner 2000; Dannenmann *et al.* 2009). The NO_3^- concentrations in roots were only in the range of 1% of total free NH_4^+ and amino acids as in other studies. As beech is a species that completely metabolises NO_3^- to NH_4^+ and amino acids in its roots before transfer to leaves as indicated by the lack of appreciable amounts of NO_3^- in the xylem (Gessler *et al.* 1998*b*), high NO_3^- availability in the soil may have led to the observed accumulation of root internal concentrations of soluble N-containing compounds, in particular to that of NH_4^+ (Fig. 3*b*). In turn, this may have caused feedback inhibition of N uptake systems (Jackson *et al.* 2008). Relief of this putative inhibition was observed when increases in soil microbial biomass resulted in decreases in soil NO_3^- concentrations. Under these conditions root NH_4^+ and free amino acid concentrations decreased and this may have induced increased activity of NO_3^- uptake systems in roots.

We can only speculate that the excessive NH_4^+ accumulation found here was a result of low C availability for further metabolism, i.e. missing C-skeletons for transamination or missing energy supply. It has been shown that the concentrations of soluble nonprotein N compounds in roots are linked to seasonal and climate-induced physiological changes in beech (Fotelli *et al.* 2002). Here, decreased concentrations of NH_4^+ and amino acids in roots after glucose application suggest that these roots had access to the external carbohydrates for increased amino acid consumption in biosynthetic processes. In fact, recycling of exuded C has been reported (Ford *et al.* 2007). Another possibility is that because microbial growth was boosted by glucose feeding, soil NO_3^- concentrations were depleted and plant N uptake was outcompeted by free-living microbes. Decreased external N availability may have caused decreases in NH_4^+ and free amino acids in roots from plants grown in glucose-treated soil. Thus, the amino acid metabolism in roots of controls might have been C-limited and that of glucose-treated plants N-limited.

Notably, girdling as well as drought stress, both of which limit accumulation of recent assimilate in and, thus, presumably C-flux to the roots, caused accumulation of amino acids, whereas this effect did not occur in drought-stressed, glucose-exposed beech roots. Drought influences not only plant C relations but has also profound effects on microbial activities (Shi *et al.* 2002; Borken and Matzner 2008). Although microbial biomass C did not decrease in response to drought, we can assume that the competitive strength of microbes for N was diminished because microbial biomass N decreased, and at the same time inorganic N, in particular NH_4^+ in the soil, increased. Since NH_4^+ and microbial biomass N were negatively correlated ($P = 0.05$), the increase in NH_4^+ concentrations in the soil may have been caused by a drought-induced decay of microbial biomass and subsequent remineralisation of dead microbial cells. Based on field observations, Dannenmann *et al.* (2009) concluded that this may afford a competitive advantage of plant roots for N uptake under water limitations. These authors found that intensive drought and drying rewetting events in a beech forest were followed by severe breakdown of soil microbial biomass. Hence, substrate availability was increased actually favouring the competitive strength of plants as indicated by increased N uptake (Dannenmann *et al.* 2009). In the present study, roots showed increased concentrations of free amino acids and NH_4^+ in response to drought, but no increase in N uptake. Since the increases in amino compounds were most pronounced when C translocation to roots was completely abolished and were not found in the presence of glucose exposure, we suggest that the changes in root soluble N compounds occur as a result of missing C for further metabolic conversion. There is, indeed, evidence that the accumulation of amino acids reflects limitations in protein biosynthesis (Good and Zaplachinski

1994). However, it needs to be pointed out that our study is laboratory based and that the applied treatments such as girdling or glucose feeding are useful to explore the metabolic potentials of root and soil microbes. Whether and under which conditions these potentials are realised in forest ecosystems will require further analyses.

Conclusion

In summary, our study shows that uptake of different N-forms by beech is controlled by various environmental factors, i.e. glutamine by drought and NO_3^- by a shift in external and internal N concentrations. We provide evidence that microbial-driven soil processes as well as root-internal processes of N transformation were strongly dependent on carbohydrates allocated from the shoot to the roots. The importance of C fluxes for the physiological continuity of the plant–soil system has been emphasised (Högberg and Read 2006). Moreover, we suggest that rhizodeposition is a key link not only for the plant–microbial C path, but also for the regulation of the plant–microbial N balance.

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Declaration

The following data shown in this thesis have been provided by other authors:

CHAPTER 4: Effects of ectomycorrhizal symbiosis on the nitrogen uptake capacities of young beech (*Fagus sylvatica*) seedlings under drought conditions, influenced by irradiance

- C, N, ^{15}N in plant samples (root, stem, leaf) were measured by Judy Simon, Chair of Tree Physiology, University of Freiburg, Germany.

Chapter 5: Girdling affects ectomycorrhizal fungal diversity and reveals functional differences of EMF community composition in a beech forest

- The carbohydrates analysis was conducted by Christine Offermann and Judy Simon, Chair of Tree Physiology, University of Freiburg, Germany.

- Meteorological data were provided by Jutta Holst, Meteorological Institute, University of Freiburg, Germany.

- Data for soil DOC and DON were taken from Dannenmann et al. 2009 (see Appendix 1).

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Publications

Winkler J B, Dannenmann M , Simon J , **Pena R** , Offermann C , Sternad W , Clemenz C , Naumann P S , Gasche R , Kögel-Knabner I , Gessler A , Rennenberg H and Polle A. **2010**. Carbon and nitrogen balance in beech roots under competitive pressure of soil-borne microorganisms induced by girdling, drought and glucose application. *Functional Plant Biology*: 879–889

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2010	Rodica Pena, Andrea Polle. Ectomycorrhizas of beech: functional diversity for N uptake under drought and effects of a direct competitor (*Cornus sp*). International workshop: Adaptation of perennial plants to episodic drought: impact on ecosystems. Goettingen, Germany, 6 - 8 October 2010. Oral presentation.
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Biodiversity: Genes, Species, Ecosystems. Giessen, Germany, 30 August - 3 September 2010. Oral presentation.

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