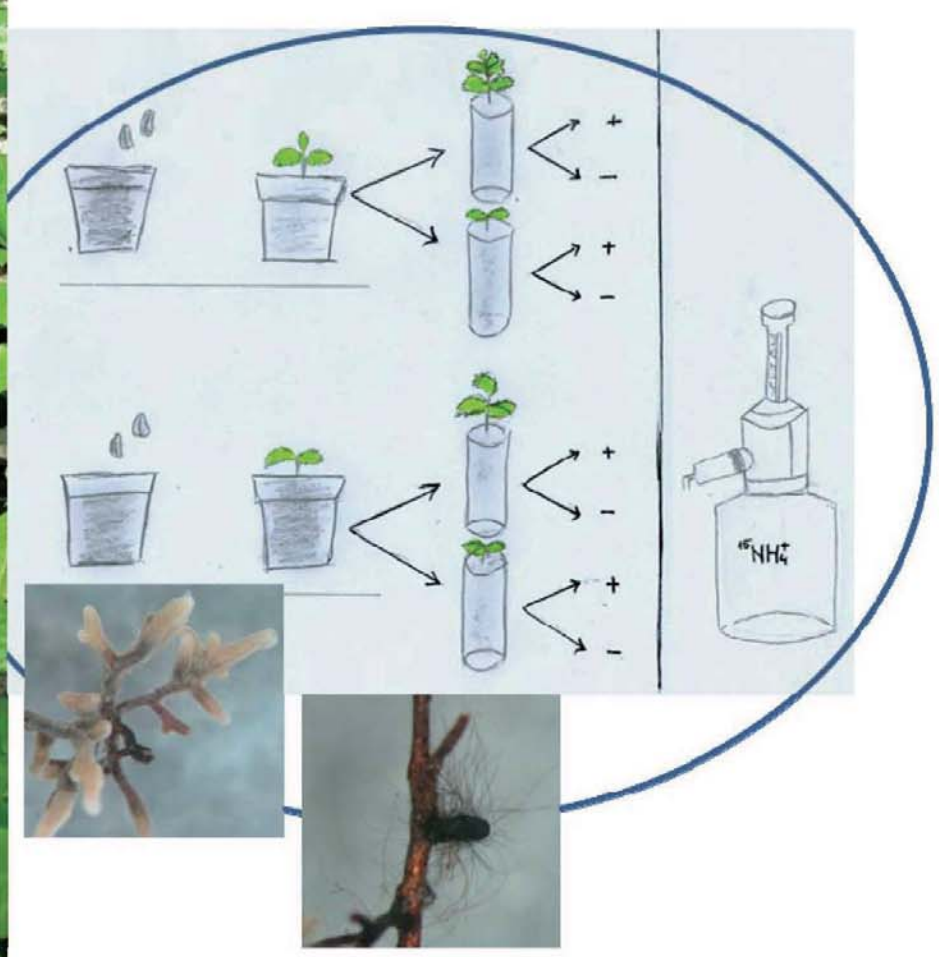
Rodica Pena

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



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

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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).

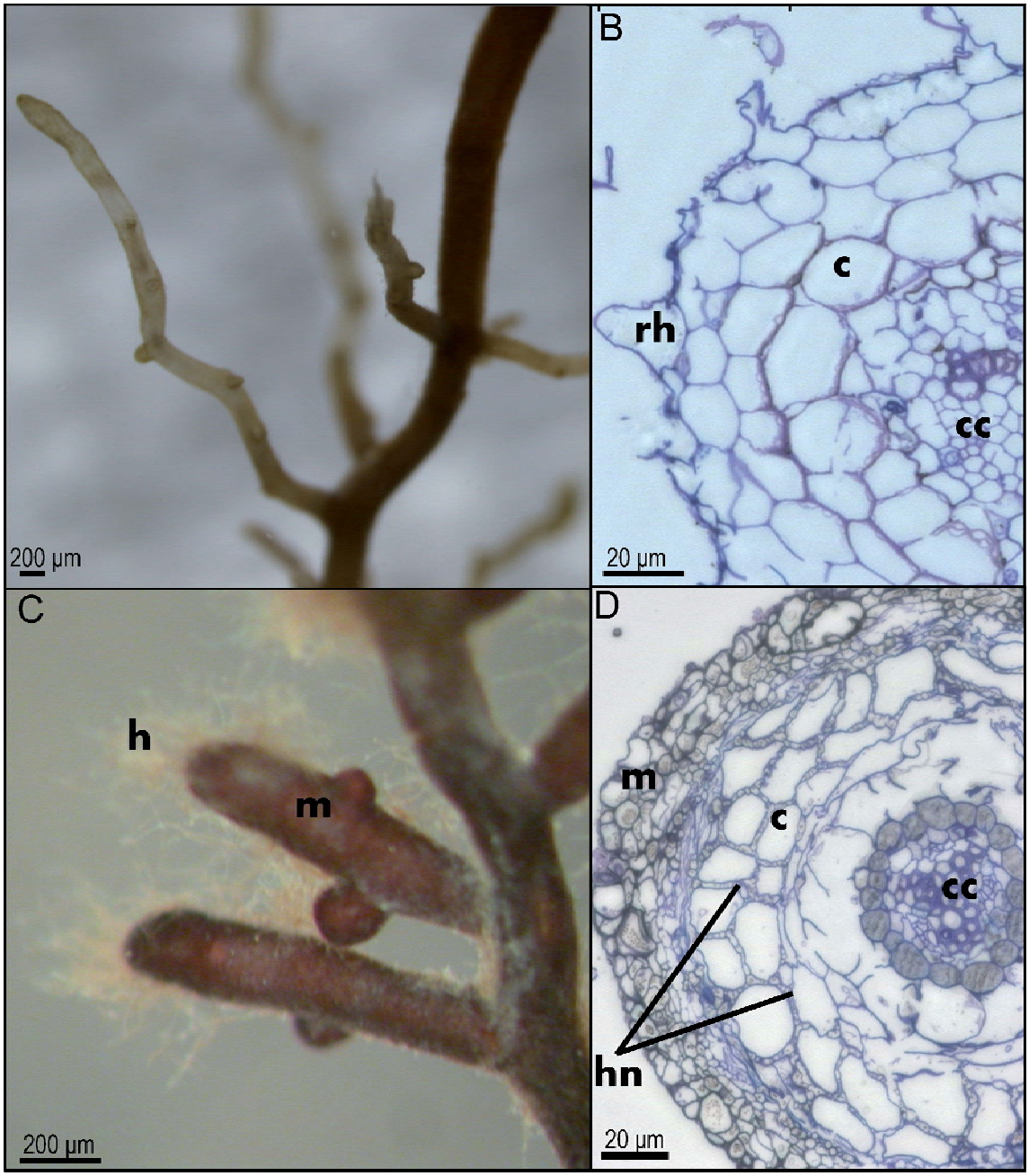

Figure 1.1: Non-mycorrhizal (A, B) and Ectomycorrhizal (C, D) root tips of young beech. Cortex cell (c), central cylinder (cc), hyphae (h), Hartig net (hn) rh, root hair (rh), mantle (m).

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