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Amoebae in the rhizosphere and their interactions with arbuscular mycorrhizal fungi: effects on assimilate partitioning and nitrogen availability for plants

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Abstract

Plants interact with multiple root symbionts for fostering uptake of growth-limiting nutrients. In turn, plants allocate a variety of organic resources in form of energy-rich rhizodeposits into the rhizosphere, stimulating activity, growth and modifying diversity of heterotrophic microorganisms.

The aim of my study was to understand how multitrophic interactions feed back to plant N nutrition, assimilate partitioning and growth.

Multitrophic interactions were assessed in a single-plant microcosm approach, with arbuscular mycorrhizal fungi (*Glomus intraradices*) and bacterial feeding protozoa (*Acanthamoeba castellanii*) as model root symbionts. Both organisms are common and abundant in the rhizosphere, contributing strongly to plant nutrient acquisition. Stable isotopes enabled tracing C (^{13}C) and N (^{15}N) allocation in the plant, into the rhizosphere and the microbial community. Microbial community composition was investigated by phospholipid fatty acid analysis.

This study offers new perspectives for the microbial loop in soil concept. Plant species identity is a major factor affecting plant-protozoa interactions. N uptake was enhanced in the presence of protozoa for *Zea mays* and *Plantago lanceolata.* The presence of protozoa increased specific root area in both plant species, whereas specific leaf area was only increased in *P. lanceolata*. *Holcus lanatus* did not respond to any parameter studied.
Protozoa in the rhizosphere mediate plant C allocation and nutrient mobilization. These responses depended on the quality of soil organic matter (assessed by C-to-N ratio of leaf litter). Plants adjusted the allocation of C resource to roots and into the rhizosphere depending on litter quality and the presence of bacterial grazers for increasing plant growth. The effect of protozoa on the structure of microbial community supplied with both, plant C and litter N, varied with litter quality.
AM-fungi and protozoa interact to complement each other for plant benefit in C and N acquisition. Protozoa re-mobilized N from fast growing rhizobacteria and by enhancing microbial activity. Hyphae of AM fungi acted as pipe system, translocating plant derived C and protozoan remobilized N from source to sink regions. This strongly affected decomposer microbial communities and processes in distance to roots. Plant growth promoting effect of protozoa in the rhizosphere fostered synergistically the exploitation of nutrients.

Major perspectives of this work will be to investigate (i) whether multitrophic interactions in our model system can be generalized to other protozoa-mycorrhiza-plant interactions (ii) whether these interactions are depending on plant phenology and plant community composition.

Key words:

Rhizosphere, carbon, nitrogen, stable isotope, ^{13}C, ^{15}N, microbial loop, multitrophic interactions, protozoa, *Acanthamoeba castellanii,* arbuscular mycorrhizal fungi, *Glomus intraradices,* microbial community, *Plantago lanceolata*, *Zea mays, Holcus lanatus*, litter quality, PLFA,

Résumé

Les interactions entre organismes du sol sont à la base de la structuration et du fonctionnement des réseaux trophiques dans le sol. Ces interactions, encore largement méconnues, sont déterminantes pour la décomposition des matières organiques, pour la nutrition des plantes et leur réponse aux attaques de bioagresseurs, au niveau des racines et des parties aériennes. Les plantes interagissent avec leurs multiples symbiotes libres et associés aux racines. Elles allouent à leur rhizosphère des composés organiques riches en énergie, les rhizodépôts, qui stimulent l'activité et la croissance des microorganismes hétérotrophes et modifient leur diversité.

L'objectif général de la thèse était de comprendre comment les interactions multi-trophiques se déroulant dans la rhizosphère agissent sur la nutrition azotée des plantes, leur croissance et sur la répartition des assimilats dans le système plante-rhizosphère-microorganismes.

Les interactions multi-trophiques sont appréhendées sur un dispositif modèle mis au point pour cette étude. Ce dernier est composé d'une plantes cultivée en microcosme dans lequel on ré-inocule le sol après stérilisation avec des espèces symbiotiques modèles : *Acanthamoeba castellanii* représentative de protozoaires bactériophages et/ou *Glomus intraradices*, espèce mycorhizienne à arbuscules. Ces deux organismes sont abondants dans la rhizosphère et leur interaction vis-à-vis de l'azote n'a pas encore été étudiée. Le cheminement de l'azote depuis la matière organique jusque dans la plante est déterminé en apportant au sol une litière foliaire préalablement marquée avec de l'azote 15 (quantifiable en spectrométrie de masse). Le carbone assimilé par photosynthèse, transporté dans la plante, libéré dans le sol par les racines et incorporé par les microorganismes est suivi grâce à la spectrométrie de masse du carbone 13 et à l'analyse en chromatographie de la composition en acides gras des phospholipides microbiens (PLFA) des communautés microbiennes. Les PLFA ont été utilisés comme un indicateur de la structure de la communauté microbienne. L'espèce végétale influence fortement les interactions entre la plante et les protozoaires. Le prélèvement d'azote (issu de la minéralisation de la litière) par la plante est stimulé pour le maïs (*Zea mais*) et le plantain (*Plantago lanceolata*). La présence de protozoaires dans le sol conduit à des feuilles moins épaisses et des racines plus fines, seulement chez le Plantain. En revanche, la houlque laineuse (*Holcus lanatus*) ne répond à la présence de protozoaires pour aucun des paramètres étudiés. L'allocation de C vers les racines et la rhizosphère et la disponibilité en azote sont influencées par la présence de protozoaires. Ces réponses dépendent de la qualité de la litière foliaire enfouie (qualité évaluée à partir du ratio C/N de la biomasse sèche). La présence de protozoaires a modifié la structure de la communauté microbienne pour la litière à C/N élevé. L'étude des interactions entre mycorhizes à arbuscules et protozoaires montre que ces 2 symbiotes présentent une complémentarité pour l'acquisition du C et de N par la plante. Les protozoaires remobilisent N à partir de la biomasse microbienne dont l'activité est stimulée. Les hyphes fongiques ne se montrent pas capables de minéraliser directement N à partir des litières. En revanche, ils transportent du C récent issu de la plante vers des sites riches en matière organique non accessibles aux racines. Ainsi, l'activité de la communauté microbienne est stimulée et la disponibilité en N augmentée lorsque des protozoaires sont présents.

Les perspectives de ce travail sont de déterminer (i) si les interactions étudiées dans ce dispositif modèle peuvent être généralisées à d'autres interactions multi-trophiques, notamment celles impliquant d'autres espèces de champignons mycorhiziens et de protozoaires (ii) si la phénologie de la plante et la composition des communautés végétales influence la nature et l'intensité des réponses obtenues.

Mots clé : Rhizosphère, carbone, azote, isotope stable, ^{13}C, ^{15}N, boucle microbienne, interactions multitrophiques, protozoaires, *Acanthamoeba castellanii,* champignon mycorhizien à arbuscules, *Glomus intraradices*, communauté microbienne, *Plantago lanceolata*, *Zea maïs*, *Holcus lanatus*, litière, PLFA

Zusammenfassung

Pflanzen interagieren mit verschiedenen Wurzelsymbionten um die Aufnahme von limitierenden Nährsoffen zu erhöhen. Im Gegenzug transferieren Pflanzen organische Ressourcen in Form von energiereichen Wurzelausscheidungen in die Rhizosphäre, die hier Aktivität und Wachstum heterotropher Mikroorganismen stimulieren und ihre Diversität fördern.

Die vorliegende Arbeit untersucht multitrophische Interaktionen in der Rhizosphäre und Ihre Auswirkungen auf Stickstoffernährung, Verteilung von Photoassimilaten und Wachstum von Pflanzen.

Die Untersuchungen erfolgten in Mikrokosmen an Einzelpflanzen. Als Modell-Symbionten wurden vesikulär-arbuskuläre (VA) Mykorrhizapilze (*Glomus intraradices*) und Bakterien konsumierende Protozoen (*Acanthamoeba castellanii*) eingesetzt. Beide Organismen sind in der Rhizosphäre von Pflanzen ubiquitär vorhanden und zahlreich. Sie tragen zu einem hohen Anteil zur Pflanzenernährung bei. Der Einsatz stabiler Isotope erlaubte die Verfolgung der Verteilung von Kohlenstoff (C) und Stickstoff (N) in der Pflanze, der Rhizosphäre und der mikrobiellen Gemeinschaft. Die Zusammensetzung der mikrobiellen Gemeinschaft wurde mit Hilfe von Phospholipidfettsäuren untersucht.

Die Studie eröffnet neue Perspektiven für das “Microbial-loop in soil”-Konzept. Die Interaktion zwischen Protozoen und Pflanzen hing von der Identität der Pflanzenart ab: In Gegenwart von Protozoen erhöhte sich die Stickstoffaufnahme von *Zea mays* und *Plantago lanceolata.* Die spezifische Wurzeloberfläche stieg bei beiden Arten an, die spezifische Blattfläche nur bei *P. lanceolata*. *Holcus lanatus* zeigte im Bezug auf die gemessenen Parameter keine Reaktion.
Protozoen in der Rhizosphäre veränderten die C-Allokation der Pflanze und die Mobilisierung von Nährstoffen in der Rhizosphäre. Die Reaktion der Pflanzen hing von der Qualität des organischen Materials im Boden ab (gemessen als C/N Verhältnis der Blattstreu). Um das Wachstum zu erhöhen, passten die Pflanzen in Abhängigkeit von Streuqualität und bakteriellen Beweidern die Allokation von C-Ressourcen in die Wurzel und Rhizosphäre an. Der Einfluss von Protozoen auf die Struktur mikrobieller Populationen, welche mit pflanzlichem C und aus der Streu stammendem N versorgt wurden, variierte mit der Qualität der Streu.
Multitrophische Interaktionen zwischen VA-Pilzen, Protozoen und Pflanzen ergänzten sich in ihrer positiven Wirkung auf Pflanzenwachstum. Protozoen re-mobilisierten N zum einen aus schnell wachsenden Rhizobakterien und zum anderen durch die Erhöhung der mikrobiellen Aktivität. Wie ein Leitungssystem transportierten die Hyphen der VA-Mykorrhiza pflanzlichen C und den von Protozoen mobilisierten N von Quellen zu Senken. Hierdurch wurde die Zusammensetzung und Aktivität der mikrobiellen Gemeinschaft in Kompartimenten, die für die Wurzel selbst nicht zugänglich waren, erhöht. Protozoen und VA-Mykorrhizen erhöhten damit in synergistischer Weise die Ausbeutung von Nährstoffen durch Pflanzen.

In Zukunft sollte untersucht werden, ob die (i) Ergebnisse der multitrophischen Interaktionen unseres Modellsystems auf andere Protozoen-Mykorrhiza-Pflanze Interaktionen übertragbar sind und die (ii) untersuchten Interaktionen von der Phänologie der Pflanze und der Zusammensetzung von Pflanzengemeinschaften abhängen.

Schlüsselwörter:

Rhizosphäre, Kohlenstoff, Stickstoff, stabile Isotope, ^{13}C, ^{15}N, Protozoa, Microbial loop, multitrophische Interaktionen, Protozen, *Acanthamoeba castellanii*, arbuskuläre Mykorrhizapilze, *Glomus intraradices*, mikrobielle Gemeinschaft, *Plantago lanceolata*, *Zea mays, Holcus lanatus*, Streu-Qualität, PLFA

Publication list

This thesis enabled to write and to present the following papers and communications:

Paper published

Koller R, Scheu S, Bonkowski M and Robin C (2008): Protozoa: small organisms in soil but strong effects on plant functioning. Seminary contribution for the Doctoral School "Ressources Procédés Produits et Environnement" (Ecole Doctorale n°410) No ISBN 2-9518564-6-6.

Papers to be submitted

Koller R, Ruess L, Robin C, Bonkowski M, and Scheu S: Effects of Protozoa on plant nutrition and carbon allocation depends on the quality litter resources in soil. Chapter 3

Koller R, Bonkowski M, Scheu S, and Robin C: Protozoa (*Acanthamoeba castellanii*) and arbuscular mycorrhizal fungi (*Glomus intraradices*) mediate the partitioning of carbon and the availability of nitrogen for *Plantago lanceolata.* Chapter 4

Koller R, Rodriguez A, Robin C, Scheu S and Bonkowski M: Protozoa and arbuscular rmycorrhiza complement each other in plant nitrogen nutrition from an organic residue patch. Chapter 5

Oral communications

Koller R, Rodriguez A, Robin C, Rosenberg K, Scheu S and Bonkowski M: Protozoa increase mineralization of nitrogen from organic patches for mycorrhizal uptake and translocation to *Plantago lanceolata* (2007): 4th meeting on Multitrophic interactions in soil, 24 – 27 June 2007, Dijon - France

Rodriguez A, **Koller R**, Robin C, Scheu S and Bonkowski M 2008: Microbial food-web interactions in the mycorrhizal plant *Plantago lanceolata,* PLANT-MICROBIAL INTERACTIONS 2008, 2-6 July, Kraków, Poland

Koller R 2008: Multitrophe Interaktionen in der Rhizosphäre: Wie Protozoen (Amöben) und arbuskuläre Mykorrhiza das Wachstum ihrer Wirtspflanze erhöhen, Invited Presentation Julius Kühn Institut Darmstadt, Germany

Posters

Koller R, Rodriguez A, Robin C, Rosenberg K, Scheu S and Bonkowski M (2007): Protozoa increase mineralization of nitrogen from organic patches for mycorrhizal uptake and translocation to *Plantago lanceolata;* Rhizosphere 2, 26-31 August 2007 Montpellier, France

Koller R, Bonkowski M, Scheu S and Robin C (2008): Complementary function of arbuscular mycorrhizal fungi and protozoa fosters nitrogen acquisition of plants (*Plantago lanceolata*), PLANT-MICROBIAL INTERACTIONS 2008, 2-6 July 2008, Kraków, Poland

Results obtained in the framework of the BIORHIZ network enabled to write and present the following paper and communication:

Ladygina N, Henry F, Kant M , **Koller R**, Reidinger S, Rodriguez A, Saj S, Sonnemann I, Witt C, Wurst S: The impacts of functionally dissimilar soil organisms on a grassland plant community are additive. (in preparation)

Medina A, Witt C, Saj S, Henry F, Bertaux J, Kant M, **Koller R**, Ladygina N, Lanoue A, Reidinger S, Rodríguez A, Sonnemann I, Wurst S, Scheu S, Baldwin I, Bonkowski M, Barea JM, Christensen S, Ferrol N, Gange A, Hedlund K, Janzik I, Mikola J, Mortimer S, van der Putten W, Robin C, Setälä H (2007): The BIORHIZ project – Who are we? Rhizosphere 2, 26-31 August 2007 Montpellier, France

Chapter I. Introduction

In total, 80-90% of plant primary production enters the soil system either as above- or belowground dead plant material ("litter") (Bardgett 2005). By recycling litter and mineralizing nutrients therein, the decomposer system provides the basis of soil fertility (Ruess and Ferris 2003). In turn, the activity and growth of soil decomposer food webs is mainly driven by litter and rhizodeposition[1] of photosynthates (Paterson *et al.* 2007). Thus, carbon (C) and nitrogen (N) fluxes between plants and decomposers is a fundamental ecosystem process and cannot be considered in isolation from each other (Prosser 2007). Mineral N limits plant growth in most terrestrial ecosystems (Vitousek and Howarth 1991) and plants interact with multiple symbionts to enhance N uptake from soil to foster growth (Clarholm 1985, Lum and Hirsch 2003, van der Heijden *et al.* 2007). However, until recent, the role of interacting symbionts on plant assimilate fluxes and N availability was scarcely investigated.

The role of multiple symbionts for plant nutrition interactions of major root symbionts are studied in this thesis. In the following, I will first introduce basic concepts on how plants interact with the microfaunal food web to enhance plant N nutrition. The regulation and functional roles of rhizodeposits are described and the model plant symbionts chosen are introduced. Finally, I will present the objectives of this thesis.

Plants manipulate the soil surrounding of roots for their own benefit (Marschner 1995). Thereby, plants influence microbial communities which are of crucial importance for plant growth, since plant N acquisition relies mainly on inorganic forms of N, i.e. the microbial conversion of organic N to inorganic forms (Hobbie 1992, Marschner 1995). Indeed, nitrate and ammonium are the major sources of inorganic nitrogen taken up by the roots of higher plants (Marschner 1995), despite plants can also use organic N sources, such as amino acids for N nutrition (Schimel and Bennett 2004, Dunn et al. 2006).

The release of C compounds by roots stimulates microbial activity. But since C-rich rhizodeposits have a high C-to-N ratio, microorganisms in the rhizosphere need other N sources like litter-N, to ensure their growth and activity (Robinson *et al.* 1989, Nguyen 2003). Subsequently, microorganisms break down litter. Depending on the availability and form of C and N available to microbial communities, mobilization (the release of N into the soil in plant available form) or immobilization of litter N (sequestering into the microbial biomass) occurs (Hodge *et al.* 2000a). Herein, the

[1] Rhizodeposition: process by which living roots release compounds into the rhizosphere

quality (C-to-N ratio) of litter mineralised by microbes is crucial (Hodge *et al.* 2000a). Bacterial grazers, such as protozoa and nematodes, graze on the microbial communities In the case of amoebae (protozoa), one third of the ingested N as NH_4^+ is excreted. Thus, *via* their grazing activity, amoebae re-mobilize the N pool locked up in bacterial biomass, thereby making it available for plant growth. This mechanism is known as "microbial loop in soil" (Clarholm 1985, Figure 1**)** and results in a marked increase in plant growth (Kuikman and Van Veen 1989, Bonkowski *et al.* 2000, Bonkowski 2004).

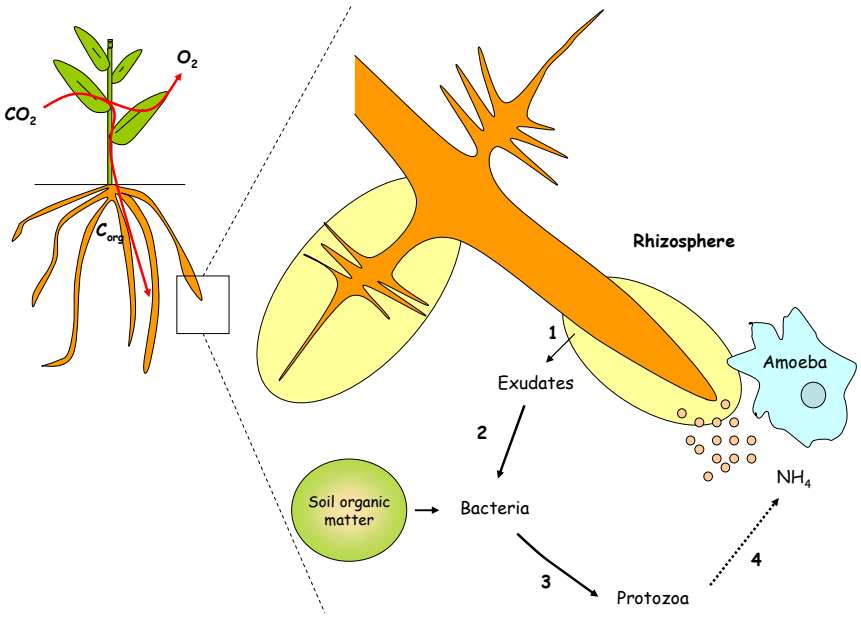

Figure 1. "Microbial loop in soil" according to Clarholm (1985): Root exudats (C_{org}) (1) stimulating growth and activity of soil bacteria (2) which sequester nitrogen (N) from organic matter in their biomass (3). Grazing of protozoa on bacterial biomass releases excess N as ammonium (NH_4^+) into the soil, which subsequently becomes available for plant uptake (4).

The "microbial-loop" mechanisms has been controversially discussed questioning whether N acquired by plant through this pathway is quantitatively significant (Griffiths and Robinson 1992, Raynaud *et al.* 2006). Additionally, it has been argued that even if the release of C promotes bacterial growth, it may consequently not induce the production of microbial enzymes for microbial decomposition of litter (Fontaine *et al.* 2003). Complementary to the microbial loop, protozoa further enhance N mineralization by increasing microbial activity (Bonkowski 2004) and altering microbial community composition (Rønn *et al.* 2002, Kreuzer *et al.* 2006). As indicated by the microbial loop, plant rhizodeposition is intimately linked to microbial functioning and consequently provides N and increases plant productivity. Thus, understanding how plants regulate C allocation to different symbionts for optimizing nutrient uptake and to satisfy their own resource requirements is important for fostering plant growth (Wamberg *et al.* 2003,

Bonkowski 2004, Matyssek *et al.* 2005, Corrêa *et al.* 2006, Kiers and van der Heijden 2006, Herdler *et al.* 2008). However, recently it has been proposed that protozoa and nematodes may also affect plant growth and root morphology by stimulating hormone production (IAA) through grazing-induced changes to the soil microbial community (Bonkowski 2004, Mao *et al.* 2007).

Regulation of carbon partitioning in the plant and rhizodeposition

Plants convert light energy into chemical energy by photosynthesis. In this biochemical process, energy of photons is used to convert CO_2 to glucose. Photosynthates are transported from "source" sites e.g. mature green leaves, to the "sink" sites. The allocation of resources among plant roots and shoots represents the largest flux of resources within a plant (Craine 2006). Therefore, the regulation of processes mediating C fixation and allocation of photosynthates are essential for growth and plant yield. A major C sink in growing plants is the roots system, which receives about 50% of the fixed photosynthates. The allocation of photosynthates to roots and into the rhizosphere is regulated by microorganisms (Brimecombe *et al.* 2001), trophic level interactions of bacteria and their grazers (Standing *et al.* 2005), plant developmental stage (Marschner 1995), soil texture (Hinsinger *et al.* 2005), N availability (Henry *et al.* 2005), light conditions and water status (Palta and Gregory 1997). However, the factors mediating belowground exchange of C between plants and soil remain poorly understood (Jones *et al.* 2004). This uncertainty is caused e.g. by the complex trophic interactions within soil foodwebs. Jones and colleagues (2004) divided the factors controlling C allocation to roots into four groups: soil as well as plant biotic and abiotic factors (Figure 2).

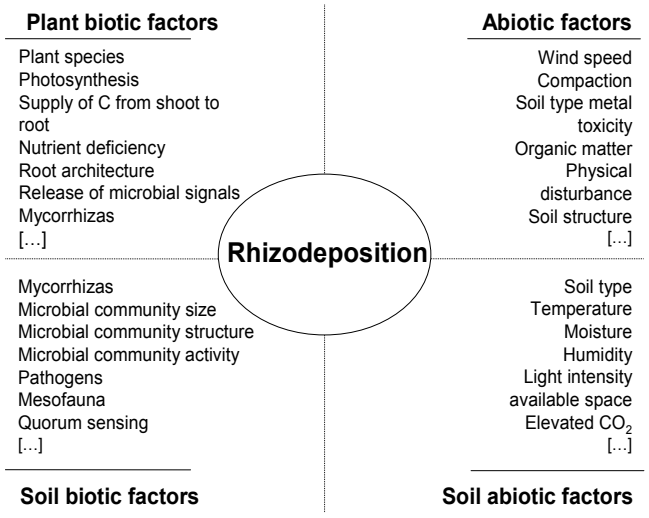

Figure 2. Schematic representation of the biotic and abiotic factors of plant and soil which influence rhizodeposition (modified after Jones et al. 2004)

Generally, rhizodeposition is estimated to be 10 to 20% of total net fixed C (Swinnen *et al.* 1994) resulting on the one hand from passive diffusion on which the plant has little control (basal exudation) and on the other hand from the controlled release of root C resources e.g. *via* ATPase co-transporters for a specific purpose in response to environmental stimuli (Jones *et al.* 2004).

I.1. Rhizodeposits: source of energy and information for microorganisms

The soil that is influenced by rhizodeposits and root activity is termed “rhizosphere” (Lynch and Whipps 1990, Hinsinger *et al.* 2005) and was first described by Hiltner (1904). Due to the release of rhizodeposits, the rhizosphere is in contrast to bulk soil not limited on C. This leads to high activity and abundance of microorganisms (Ekelund and Ronn 1994). Concentration of microbes in the rhizosphere can reach 10^{12} per gram of rhizosphere soil as compared to < 10^{8} in the bulk soil (Foster 1988). Subsequently numbers of bacterial feeders like protozoa are increased (Clarholm *et al.* 2006). As a result fast growing bacteria, typically colonizing this zone around the root (Alphei *et al.* 1996, Bonkowski *et al.* 2000), are strongly top down controlled by amoebal grazing (Wardle *et al.* 2004, Griffiths *et al.* 2007) (see above).

Plant roots are chemical factories that synthesize a wide variety of secondary metabolites which are biologically active and may act as messengers orientating interactions between roots and rhizosphere organisms (Standing *et al.* 2005, Bais *et al.* 2006, Prosser 2007). Soluble rhizodeposits contain a variety of monomeric compounds like sugars, amino acids and fatty acids, but in particular carboxylic acids like citrate, malate, succinates and oxalate are abundant and important for bacterial growth (Sørensen and Sessitsch 2007). Generally very little is known about chemical composition of rhizodeposits in soil, but Teplinski *et al*. (2000) demonstrated that plants can release compounds that interfere the communication of gram^{-} bacteria in the rhizosphere (N-acyl homoserine lactones like compounds, autoinducer signals).

The flux of molecules in the rhizosphere is bidirectional (Jones and Darrah 1996, Phillips *et al.* 2003). Indeed, roots may act as both, source (efflux of organic compounds) and a receptor of rhizodeposits (re-uptake of root released compounds (Phillips *et al.* 2004). Thus, plants may act as an important base for controlling the soil food web and /or a component of the molecular control points for the co-evolution of plants and rhizosphere organisms (Cheng and Gershenson 2007).

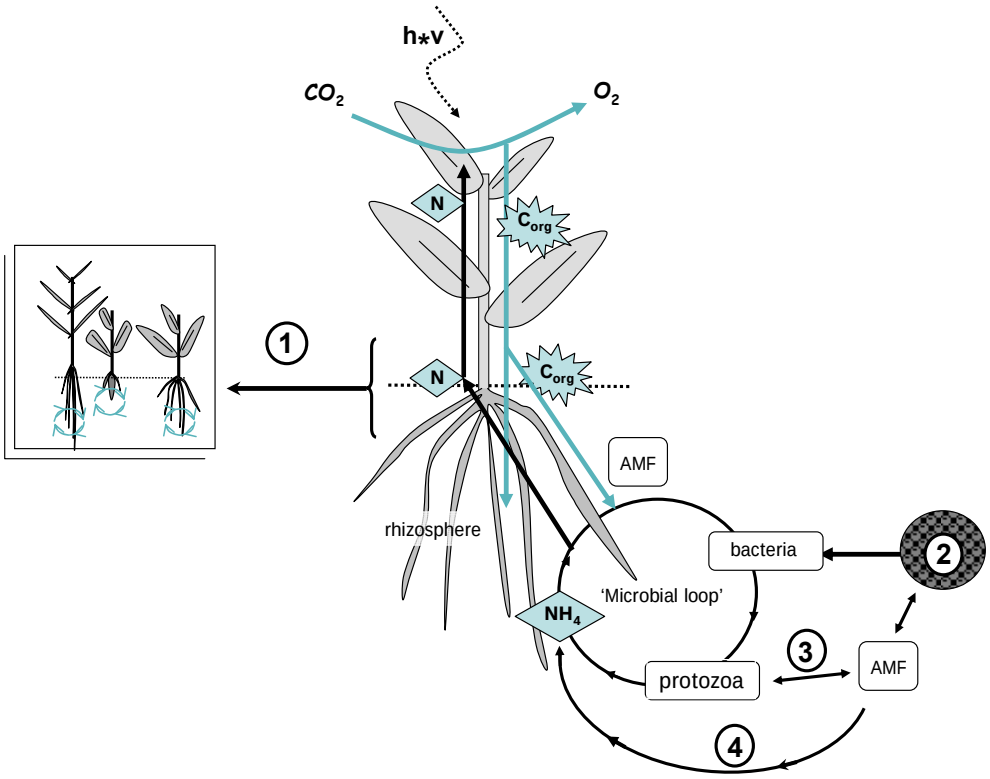

Figure 3. Multitrophic interactions in the rhizosphere. In the 'microbial loop', plant derived carbon (C) fuels microbial activity and mineralization from litter. Protozoa enhance plant growth by nutrient re-mobilization from grazed bacterial biomass and by positively affecting microbial community structure. I tested whether these mechanisms are modified by plant species (1) or litter quality (2). Arbuscular mycorrhizal fungi (AMF) are known to enhance plant growth by enhancing absorptive root area. I examined whether protozoa and AMF interact to shift C allocation and foster plant nutrient acquisition in the presence (3) and absence of roots (4).

I.2. Photosynthates allocation towards root infecting and free living symbionts: AM fungi and protozoa

From a plant perspective a tight control over its C budget should exist since lost C does not contribute to dry matter production. Contrary, from the microbial perspective, mechanisms to increase the net efflux of C from roots are likely to occur to maintain growth and activity (Bonkowski 2004). Even though plants transfer large amounts of photosynthates into the rhizosphere, competition for plant C between different root symbionts has been suggested (Vierheilig *et al.* 2000, Phillips *et al.* 2003). Competing symbionts can be separated into root infecting and free living microorganisms. We chose arbuscular mycorrizal (AM) fungi as a root infecting symbionts (Phylum Glomeromycota) (Schüßler *et al.* 2001). AM fungi form symbiosis with about 80% of all terrestrial plant genera (Smith and Read 1997) and about 60% of all land plants end up into symbiosis with arbuscular mycorrhizal fungi (Trappe 1987). Moreover AM fungi are suggested to be the oldest and most important terrestrial plant mutualists (Brundrett 2002). All mycorrhiza types possess two common features: an interface between plant root and fungal cells and extraradical hyphae extending into the soil (Johnson and Gehring 2007). Dead and living biomass of extraradical parts of AM fungi (e.g. spores and hyphae) make up 5 to 50 % of microbial biomass in agricultural soils (Olsson *et al.* 1999). AM fungi contribute predominantly to host plant phosphorous acquisition by increasing the absorptive area of roots *via* extraradical hyphae (Smith and Read 1997). In exchange for providing plants with mineral nutrients, the obligate biotroph AM fungi receive up to 30% of recently fixed plant assimilates (Johnson *et al.* 2002, Nguyen 2003, Jones *et al.* 2004, Heinemeyer *et al.* 2006). Thus, the mycelial system provides vital conduits for the translocation of nutrients from soil to plants and for reciprocal transfer of C from plant roots into extraradical AM hyphae (Smith and Read 1997). Recent studies demonstrate that AM fungi contribute to plant N gain by colonising litter patches (Hodge *et al.* 2000b, Hodge *et al.* 2001). However, there is no evidence that AM fungi have significant saprotrophic ability (Smith and Read 1997) and it is still unclear to what extend N allocation by AM fungi improves host plant performance (Johnson *et al.* 1997). The direct and rapid acquisition of photosynthetically fixed C and the rapid turnover of hyphal networks in soil (Staddon *et al.* 2003) suggest that mycorrhizal fungi form significant agents sequestering C in soil (Staddon 2005). The association between plant and AM fungi is mediated by the availability of nutrients, e.g. depends on the availability of soil N (Johnson and Gehring 2007). Moreover, plant growth responses on AM fungi association ranges in a continuum from positive

(mutualism) to neutral (commensalism) and even to be negative (parasitism) (Johnson *et al.* 1997), but it is assumed that mutualism dominates (Marschner 1995, Smith and Read 1997). Species identity of the fungal partner determines plant nutrient supply that can directly affect plant growth (van der Heijden *et al.* 2003) and competitiveness of coexisting plant species. Additionally, terrestrial ecosystems contain many AM fungi and plant species that coexist in communities (Johnson *et al.* 1991, Allen et al. 1995, Sanders *et al.* 1996, Helgasson *et al.* 1998, Picone 2000, Ergeton-Warburton and Allen 2000). This indicates that the influence of AM fungi on plant growth is complex and, as shown recently, plant species and genotypes may vary in their responses to mycorrhizal colonization (Rillig *et al.* 2008). In conclusion, AM fungi contribute to complex belowground interactions and their activity influence the functioning and activity of other soil organisms that feed back to plant performance.

We have selected protozoa as a free living root symbiont and strong bacterial grazers. Protozoa are crucial in mediating rhizosphere food webs and plant performance. Nutrient decomposition by bacteria and fungi is a function of litter quality with the mobilization of nutrients locked up in microorganisms being a function of microbial feeding fauna, e.g. the microbial loop in soil (see above) (Bonkowski *et al.* 2000, Hodge *et al.* 2001). Next to a range of microbes (e.g. slime molds, primitive algae) 'protozoa' are belonging to the paraphyletic "protista"-group that consists of eukaryotes that are not animals, true fungi or green plants (Clarholm *et al.* 2006). About 200,000 protist species (Clarholm *et al.* 2006) and about 40,000 protozoan species are named (Coûteaux and Darbyshire 1998). From soil about 400 species of ciliates, 260 species of heterotrophic and autotrophic flagellates, 200 species of testate amoebae and 60 species of naked amoebae has been reported (Foissner 1996). From a 'traditional" point of view protozoa can be divided into the following groups:

- Flagellates (4-15 µm body length)
- Ciliates (20-600 µm body length)
- Amoebae: testate (20-80µm) and naked (15-100 µm; but only 1 µm thick) (Clarholm *et al.* 2006).

In addition, protozoa may be grouped according to feeding preference such as photoautotrophs, bacterivores/detritivores, saprotrophs, algivores, non-selective omnivores and predators (Coûteaux and Darbyshire 1998). This grouping, however, is not related to phylogeny and relates little to ecological functions (Adl and Gupa 2006). The number of protozoa in soil varies between 10,000 and 100,000 individuals (active and encysted) per gram of soil with the numbers increasing with organic C and N (Clarholm *et al.* 2006), and soil moisture (Clarholm 2004), and decreasing with soil

depth (Adl and Gupa 2006). Active protozoa often are enclosed in a thick cell membrane but a cell wall is missing. This makes them dependent on soil water films (Clarholm *et al.* 2006). The life cycle of terrestrial protists contains a resting ("cyst") stage allowing to survive adverse conditions, e.g. dry periods (Ekelund and Rønn 1994, Clarholm *et al.* 2006). As a consequence soil protist communities consist of active and inactive individuals. Therefore, the local microenvironment and microclimate is an important factor driving activity and abundance of protists (Adl and Gupa 2006). Heterotrophic protozoa have to obtain their organic C and energy from their environment either by absorbing dissolved organic substances (osmotrophy) or by ingestion of organic material by phagocytosis (Ekelund and Rønn 1994). Recently, authors showed that a mucilage-border cells-complex facilitates beneficial effects of protozoa on plant growth (Somasundaram *et al.* 2008). Besides predatory bacteria, protozoa and bacteria form the oldest predator-prey relationship and protozoa have the capacity to strongly influence the bacterial community structure in soil (Rønn *et al.* 2002, Rosenberg 2008). Protozoa select prey species according to size, cell wall chemistry, nutritional value and toxic or inhibitory compounds (Simek *et al.* 1997, Jousset *et al.* 2006). Naked amoebae form the dominant group of soil protozoa reaching up to 2,000,000 ind. g^{-1} dry weight of soil (Curl and Harper 1990). With their tiny and flexible pseudopodia they are able to exploit prey in micropores of a diameter of only 1 µm (Foissner 1999). In agricultural soils, naked amoebae are the most competitive bacterial feeders at high bacterial densities and exert strong top-down control on bacterial populations (Clarholm *et al.* 2006).

I.3. Protozoa - arbuscular mycorrhizal fungi interactions

Although plants interact with multiple root infecting and free living symbionts to meet their need for mineral N (van der Heijden *et al.* 2007), little is known on how multiple plant-mutualist interactions feed back on plant nutrition (Jentschke *et al.* 1995, Wamberg *et al.* 2003, Herdler *et al.* 2008). Hence, most studies investigating plant - symbiont interactions only consider pairwise interactions. This, however, is likely to underestimate the overall beneficial effect of symbionts, since symbionts may differ fundamentally and interact synergistically (Stanton 2003, Wamberg *et al.* 2003, Strauss and Irwin 2004). I focused on protozoa – AM fungi interactions since AM fungi are ubiquitous forming part of virtually any plant rhizosphere and therefore are of significant importance for plant nutrition and growth (Hodge *et al.* 2001, Bonkowski 2004). Yet, only few studies investigated protozoa - mycorrhizia interactions. The first studies on Protozoa – mycorrhiza interactions focused on ectomycorrhizal fungi (Jentschke *et al.* 1995, Bonkowski *et al.* 2001) and documented contrasting effects of protozoa and

mycorrhizal fungi on root morphology and mineral nutrition of spruce seedlings. The presence of mycorrhiza led to a shorter, less-branched root system, whereas protozoa decreased the length of fungal mycelia in soil, but increased the root surface. Moreover, mycorrhiza enhanced P uptake by plants, while protozoa increased N mobilization. The simultaneous presence of AM fungi and protozoa increased plant N and P nutrition compared to control treatments without mycorrhiza and protozoa (Bonkowski *et al.* 2001). The authors speculated that plants allocate C resources to optimize simultaneous exploitation of both mutualistic relationships. Yet, studies investigating plant C allocation patterns in presence of both protozoa and mycorrhizal fungi are lacking.

I.4. Objectives

The objective of this PhD thesis is to examine how biotic interactions in the rhizosphere feed back to plant C assimilation, partitioning and rhizodeposition. Moreover, I investigated the consequences of plant interactions with root infecting and free living microbial symbionts on the bioavailability of nitrogen and the role of litter quality (C-to-N ratio) for these interactions (Figure 2).

The objective of the first experiment in this study was to establish a model system allowing to investigate plant - microfaunal food web interactions as suggested by the microbial loop concept **(Chapter 2)**. In addition, the experiment tested whether beneficial effects of protozoa on plants vary with plant species. Plant species with strongest responses were chosen as model plants for following experiments.

The second experiment of this study evaluates how litter quality (as indicated by litter C-to-N ratio) mediates the mobilisation of nutrients by amoebae and for the feedbacks to plants via the microbial loop in soil **(Chapter 3)**. Feedbacks on plant C assimilate partitioning but also on the structure of the microbial rhizosphere community were examined.

The third and fourth experiment evaluates whether root infecting and free living plant symbionts interact in affecting plant N acquisition and plant growth. Furthermore, we evaluated whether the presence of both symbionts mediate photoassimilate partitioning and whether plant derived C is allocated to optimize the simultaneous exploitation of both mutualistic relationships by plants. This was evaluated for homogeneously distributed litter resources in soil. **(Chapter 4)**. To obtain a more mechanistic understanding of the processes involved in N mobilization from litter, protozoa - AM fungi interactions were investigated by litter materials in a patch separated from direct access by plant roots **(Chapter 5)**.

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Chapter II. The impact of protozoa on plant nitrogen uptake and morphology varies with plant species

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Summary

- Nitrogen (N) is the major limiting nutrient for plant growth in terrestrial ecosystems. Plants invest high amounts of photosynthates into the root zone ("rhizosphere") to enhance microbial mineralization of N. Protozoa foster N uptake by re-mobilizing N from bacterial biomass ('microbial loop').

- We performed a growth chamber experiment to evaluate whether the effect of Protozoa (*Acanthamoeba castellanii)* on plant growth and plant morphology varies with plant species. Plant N uptake from litter was traced by adding ^{15}N labelled *Lolium perenne* leaf litter to the soil.

- N uptake and morphology of the investigated plant species (*Holcus lanatus, Zea mays, Lotus corniculatus* and *Plantago lanceolata*) indeed varied in presence of protozoa. *H. lanatus* was not affected by protozoa, whereas *Z. mays* increased its specific root surface and *P. lanceolata* increased both the specific leaf and root surface. We found an enhanced N uptake from added litter in presence of amoebae for *Z. mays* only.

- We conclude that plant species identity has to be considered in plant-protozoa interactions.

II.1. Introduction

Plants manipulate the soil surrounding roots for their own benefit, in particular to increase nutrient capture (Marschner 1995). Nitrogen (N) is the major limiting element for plant growth in terrestrial ecosystems (Vitousek and Howarth 1991, LeBauer and Treseder 2008). Since plants rely mainly on inorganic forms of N, they depend on the mineralization of N, i.e. the microbial conversion of organic N to inorganic forms (NO_3^- and NH_4^+) (Hobbie 1992, Marschner 1995). Hence, plants interact with multiple symbionts to enhance nutrient uptake from soil and subsequent growth (van der Heijden *et al.* 2007). To achieve this goal, plants allocate about 20% of their net fixed C into rhizosphere soil to stimulate growth and activity of heterotrophic microorganisms (Grayston *et al.* 1998, Lu *et al.* 2004). Due to high C-to-N ratio of rhizodeposits (Robinson *et al.* 1989, Nguyen 2003), microbes need other N sources to ensure their growth and activity by litter decomposition that foster bioavailability of mineral N.

Litter materials entering the soil often exceed a C-to-N ratio of 30 (Kaye and Hart 1997). When substrates of high C-to-N ratio are decomposed, most litter N is sequestered into microbial biomass (Hodge *et al.* 2000). The rate of N mobilization and subsequent plant uptake is driven by the interplay between microbes and its microfaunal grazers (Bonkowski 2004, Scheu *et al.* 2005). Microbial grazers, such as protozoa, mobilize the microbial fixed N by excreting one third of consumed N from bacterial biomass into the soil thereby making it available for plant uptake. This mechanism is known as “microbial loop in soil” (Clarholm 1985, Bonkowski 2004). Additionally, it has been proposed that protozoa stimulate plant growth and changes root morphology by stimulating plant growth-promoting rhizobacteria (Bonkowski and Brandt 2002). Thus, from the plant perspective protozoa function as bacteria-mediated mutualists: they promote plant growth by (1) mobilizing nutrients fixed in bacterial biomass and (2) favouring beneficial bacteria.

Plant species likely differ in the way and extent they modulate the structure and activity of soil microorganisms (Marschner 2001, Garland 1996). Differences between plant species has been attributed to variations in root exudates (Garland 1996, Grayston *et al. 1998,* Bardgett *et al. 1999,* Smalla *et al. 2001)*. Indeed, the composition of root exudates vary among plant species and temporally and spatially varying substrate availability is known to be a key factor affecting the community structure of rhizosphere microorganisms (Grayston *et al.* 1998, Griffiths *et al.* 1999, Baudoin *et al.* 2003, Butler *et al.* 2003, Nguyen 2003). This suggests that plant specific traits may affect the

'microbial loop' and ultimately feed back to plant N uptake (Griffiths *et al.* 2007). The ability of plant species to respond to environmental conditions, i.e., the plasticity of foraging for nutrients, varies among plant species (Grime 1979). Differential responses may be based on changes in root morphology but also in the composition of root exudates (Grime 1979, Campbell *et al.* 1991).

The objectives of the experiment were:

- to establish a model system allowing to investigate the importance of the microbial loop in soil for plant resource acquisition;

- to evaluate if plant responses to the presence of protozoa (*Acanthamoeba castellanii)* in the rhizosphere varies with plant species.

We hypothesize that grazing on microorganisms by protozoa (1) alters plant growth and the surface area of plant roots, and (2) modulates the uptake of ^{15}N from litter in soil, with the effects varying among plant species.

We performed a growth chamber experiment to evaluate N availability for four plant species: two grasses (*Holcus lanatus*, C_3, and *Zea mays*, C_4), a herb (*Plantago lanceolata*) and a forb (*Lotus corniculatus*). *Holcus lanatus*, *P. lanceolata* and *L. corniculatus* coexist at the site where the soil for the experiment was taken and are used as model plants in the BIORHIZ project. *Zea mays* is a model plant in rhizosphere research in the Nancy lab. We added ^{15}N labelled *L. perenne* litter to the soil to allow tracing N transfer from litter to plant. Plant growth and leaf and root morphology were assessed.

II.2. Materials and Methods

II.2.1. Plants, microcosms and incubation procedure

Three plants of different functional groups that are common and widely distributed in Europe, were selected as model plants (*H. lanatus*, *L. cornicularius* and *P. lanceolata*). Additionally, *Z. mays* (DEA, Pioneer France) was selected as an important culture plant in Europe and a model plant for rhizosphere research.

After surface sterilization (Benizri *et al.* 1995), seeds were transferred aseptically in Neff's Modified Amoeba Saline (NMAS) (Page 1976) mixed 1:9 (volume:volume) with nutrient broth (NB) (Merck) (NB-NMAS) (100 µl) in 96' well micro-titer-plates (Grainer, Germany) (except of *Z. mays* which was directly transferred on NB-NMAS Agar). Here seeds were allowed to germinate in the dark at 20°C. After germination, seedlings were

transferred on 10% NB-NMAS Agar in Petri dishes and cultured at 21°C before aseptical transfer into the microcosms.

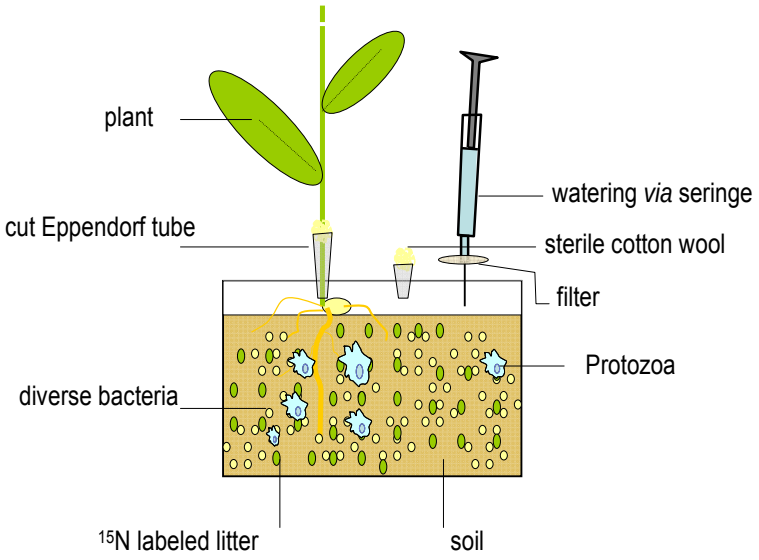

Figure 4. Microcosm set up: model plants (*Holcus lanatus, Zea mays, Lotus corniculalus* and *Plantago lancelata*) were grown in soil inoculated with protozoa free microbial rhizosphere microorganisms. The Amoeba treatment received axenic *Acanthamoeba castellanii* as microfaunal grazer. Soil was protected from airborne cysts of protozoa by wrapping sterile cotton wool around the basis of the shoot. To follow nitrogen uptake from soil into model plants ^{15}N labelled *Lolium perenne* litter was added to the soil.

II.2.2. Preparation of microcosms and soil

Soil was collected from the upper 20 cm of a grassland site grown on a former agricultural field, which had been abandoned for more than 10 years (Van der Putten *et al.* 2000). The soil was taken in autumn and stored at 4 °C before sieving (4 mm) and use in the experiment. It contained 21.3 g kg^{-1} organic carbon, 1.27 g kg^{-1} total N, 0.33 g kg^{-1} total P and had a pH of 6.3.

^{15}N labelled *Lolium perenne* leaf litter was produced as described by Wurst (2004). Before autoclaving, ^{15}N labelled *L. perenne* leaf litter (C-to-N ratio 8.2) was homogeneously mixed with non labelled *L. perenne* litter (C-to-N ratio 11.5) to achieve litter containing 10 atom% ^{15}N. To 250 g dry weight soil 0.39 g of the litter was added and mixed homogeneously. Prior to transfer of the soil into the microcosms it was autoclaved three times (20 min each, 121°C). Microcosms consisted of 250 ml polypropylen pots with a circular opening for plant shoots in the lid. Openings were sealed with sterile cotton wool to avoid contamination by airborne cysts of protozoa. A second opening was installed to improve aeration of the system (Figure 4).

II.2.3. Inoculation with bacteria and protozoa

A natural protozoa-free soil bacterial inoculum[2] was prepared from the upper 20 cm soil from a grassland site grown on a former agricultural field, which had been abandoned for more than 10 years (van der Putten *et al.* 2000). The supernatant of a soil slurry (50 g fresh weight soil mixed 1:1 with NMAS on a horizontal shaker at 70 rpm for 20 min) was passed consecutively through two filters of 3 µm and then 1.2 µm (Bonkowski and Brandt 2002). The protozoa-free filtrate was cultured in NB-NMAS and checked for contamination for seven days by microscopic observation. Additionally one third of the filtrate was cultured in autoclaved soil. Both cultures were stored at 21°C in a climate chamber.

Prior adding 2 ml of protozoa-free filtrate to the microcosms, 5 g of the protozoa-free bacteria-soil culture was added into sterile soil of each microcosm. Subsequently, the soil was compressed to a density of 1.3 g cm^{-3} and incubated for 1 week at 24°C and 75 % relatively humidity in a climate chamber. Then, 0.78 g glucose was added to each pot in 3 ml aqueous solution to stimulate microbial activity. Prior to the addition of amoebae, small amounts of soil (covering the tip of a spatula) of each pot were mixed with NB-NMAS and checked for contamination for 7 days.

Axenic amoebae (*Acanthamoeba castellanii*) (Rosenberg 2008) were prepared following a modified protocol described by Bonkowski & Brandt (2002). Briefly, the amoeba culture was washed and centrifuged twice in NMAS (1000 rpm, 2.5 min). Protozoan treatments received 1 ml (approximately 8000 individuals) of the protozoa suspension, whereas the control treatments received 1 ml NMAS.

II.2.4. Plant transfer and cultivation

Seven days after protozoa inoculation, plants of similar size were selected and transferred into the microcosms under sterile conditions. Microcosms were then incubated in a climate chamber (18°C / 22°C night/ day temperature, 70% of humidity, 14 h of photoperiod, 460 ± 80 µmol m^{-2} s^{-1} photon flux density in the PAR range at plant level). Soil moisture was gravimetrically maintained at 70% of the water holding capacity by watering with sterile distilled water using a 0.02 µl syringe filter. Plant shoots were fixed in the opening of the microcosms with sterile cotton wool to avoid contamination with protozoa by air borne cysts.

[2] Bacterial inoculum may have contained other soil organism than bacteria, e.g. spores of soil fungi

II.2.5. Harvesting and analytical procedures

Plants were destructively harvested 21 days after transfer into the soil except for *Z. mays* which was harvested after 16 days to avoid root growth limiting conditions in the microcosms.

Plant leaf and root surface was scanned and analysed by WinFolia and WinRhizo software (Régent Instruments, Ottawa, Canada), respectively. Plant materials were subsequently freeze dried for biomass determination. Root adhering soil was taken as rhizosphere soil and separated from roots by handpicking. Subsamples of adhering soil were dried for water content determination (80°C, 48 h). Mineral N content was determined from 6 g root free adhering soil subsamples by extracting with 50 ml 0.5 M K_2SO_4 for 1 h at 130 rpm min^{-1} and subsequent filtering. Extracted samples were kept frozen until analysis. Mineral N (N_{min} = NO_3^-N + NH_4^+-N) content of the K_2SO_4 extracts and measured in a Traax 2000 analyser (Bran and Luebbe).

Plant tissue and soil samples were milled to fine powder for analysis of total plant C and N as well as $^{15}N/^{14}N$ ratio by an elemental analyser (Carlo Erba, Na 1500 type II, Milan, Italy) coupled with an isotope mass spectrometer (Finnigan Delta S, Bremen, Germany). Data were presented as excess ^{15}N compared to the natural abundance.

Total numbers of protozoa were enumerated by the most probable number technique (Darbyshire *et al.* 1974). Briefly, 5 g of soil were dispersed in 20 ml NMAS and shaken for 20 min at 75 rpm. Aliquots of 0.1 ml were added to microtiter plates and diluted two fold in 50 µl sterile NB-NMAS. Microtiter plates were incubated at 15°C and counted every second day for 21 days until protozoan numbers remained constant. Numbers were calculated according to Hurley and Roscoe (1983).

II.2.6. Statistical analysis

The effect of protozoa on the mobilization of N, plant N uptake and morphology of roots and leaves was analysed separately for each plant species with Amoeba as factor in SAS (v. 9.1) (n=7 for *Zea mays* and n=4 for *H. lanatus* and *P. lanceolata*, n=5 for bare soil). Normal distribution and homogeneity of variance were improved by log-transformation (Sokal and Rohlf 1995).

II.3. Results

The model microcosm system successfully protected protozoa contaminations from airborne cysts; no protozoan contaminations were found in the treatments. Due to poor establishment after transplanting, probably caused by the switch from axenic to microcosm conditions, only 4 of the initially 8 replicates per treatment could be used for *P. lanceolata* and *H. lanatus* and 7 for *Z. mays*. *Lotus corniculatus* did not establish after transfer of young seedlings into the microcosms. This might have been due to low mineral N in the soil and insufficient N_2 fixing symbiotic bacteria (*Rhizobia* spp.) in the re-established microbial community (Lum and Hirsch 2003, Wurst and van Beersum 2008).

II.3.1. Plant growth as affected by Acanthamoeba castellanii

Protozoa did not significantly affect leaf and root biomass in the tested plant species (Table 2, Table 3). Specific root area of *P. lanceolata* and *Z. mays* increased in presence of amoebae by factors of 2.1 and 1.7, respectively (Figure 5). Additionally, the specific leaf surface of *P. lanceolata* increased 1.3-fold in presence of amoebae (Table 3, Figure 5). Generally, specific root area was lower for *Z. mays* than in *H. lanatus* and *P. lanceolata* indicating bigger and more compact roots of *Z. mays*. *Holcus lanatus* had the finest root system of tested plant species.

Plant species did affect the total numbers of *A. castellanii* in soil but the number peaked in planted soils where numbers were increased from 1089 ± 920 to 20185 ± 9184, respectively at the end of the experiment ($F = 25.8$, $p < 0.0001$). Mineral N concentration in rhizosphere soil was highly increased 2.7-fold in the presence of protozoa in bare soil but remained unaffected in planted soils (Table 2, Table 3).

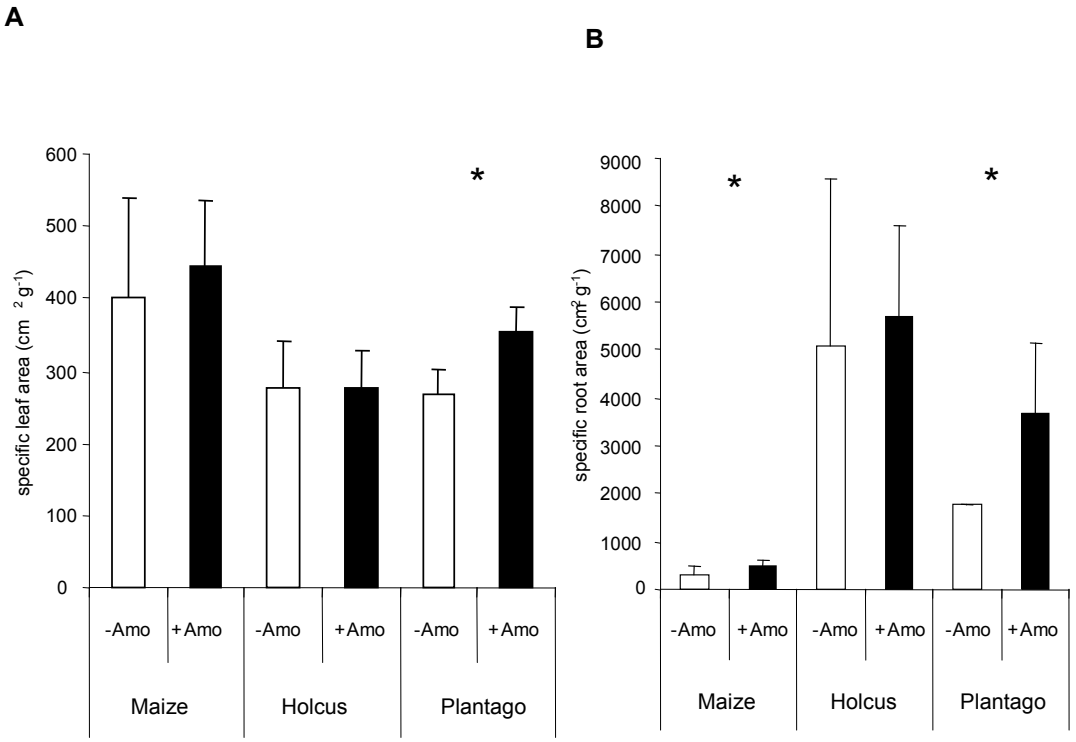

Figure 5. Specific leaf **(A)** and root surface **(B)** of *Zea mays* (Maize), *Holcus lanatus* (Holcus) and *Plantago lanceolata* (Plantago) in the presence (+Amo) and absence (-Amo) of *Acanthamoeba castellanii*. Means+1SD; * p≤0.05.

The tissue C-to-N ratio of plants did not differ between plant species in the presence of amoebae (Table 2, Table 3). In the presence of protozoa concentration of ^{15}N was 1.2 fold higher in leaves and 1.6 fold in roots of *Z. mays* as compared to the control. Plant's ^{15}N uptake from total ^{15}N added to soil was lowest for *H. lanatus* and *P. lanceolata* in the absence of Amoeba and highest for *Z. mays* in the presence of amoebae (Figure 6, Table 3). The presence of amoebae did not increase parcentage ^{15}N uptake from total added litter-N for *H. lanatus* (F 0.224, p = 0.656), *P. lanceolata* (F = 1.483, p = 0.29) but for *Z. mays* (F 7.74, p = 0.024) (Table 1).

Table 1. ^{15}N taken up by *Zea mays* (Maize), *Holcus lanatus* (Holcus) and *Plantago lanceolata* (Plantago) from ^{15}N added to soil (percentages of total)

Maize		**Holcus**		**Plantago**	
-AMO	+AMO	-AMO	+AMO	-AMO	+AMO
4.7±2.83	12.2±60	1.15±0.78	1.7±1.4	2.9±0.68	4.7±2.65

Table 2. Root and leaf biomass, tissue C-to-N ratio of *Zea mays* (Maize), *Holcus lanatus* (Holcus) and *Plantago lanceolata* (Plantago), and miner nitrogen in soil in the absence (-AMO) and presence (+AMO) of Protozoa (*Acanthamoeba castellanii*) in soil (means ± SD)

	Maize		**Holcus**		**Plantago**		**Bare soil**	
	-Amo	+Amo	-Amo	+Amo	-Amo	+Amo	-Amo	+Amo
Leaf [g plant^{-1}]	0.24±0.113	0.26±0.081	0.11±0.082	0.20±0.113	0.16±0.156	0.11±0.055	-	-
Root [g plant^{-1}]	0.15±0.031	0.17±0.051	0.03±0.035	0.07±0.115	0.07±0.054	0.03±0.012	-	-
C-to-N ratio	22.65±3.855	17.21±4.289	14.31±1.001	16.56±1.829	17.75±6.43	12.84±2.75	-	-
N_{min} [mg*g^{-1} soil^{-1}]	0.26±0.253	0.48±0.574	0.34±0.349	1.21±1.633	0.22±0.238	0.40±0.237	0.36±0.119	0.79±0.341

Table 3. F- and p-values of effects of amoebae on traits of *Zea mays* (Maize), *Holcus lanatus* (Holcus) and *Plantago lanceolata* (Plantago), and on mineral nitrogen in soil; significant differences are labelled in bold

	Maize		**Holcus**		**Plantago**		**Bare soil**	
	F	**p**	**F**	**p**	**F**	**p**	**F**	**p**
leaf biomass	0.82	0.38	2.41	0.15	0.27	0.63	-	-
root biomass	1.04	0.33	2.85	0.15	0.58	0.48	-	-
specific leaf area	0.84	0.38	0.14	0.72	**10.20**	**0.03**	-	-
specific root ara	**8.21**	**0.02**	0.67	0.44	**13.17**	**0.02**	-	-
C-to-N	2.99	0.11	6.79	0.06	1.57	0.24	-	-
excess ^{15}N leaf	**5.70**	**0.03**	1.99	0.19	2.01	0.23		
excess ^{15}N root	**4.85**	**0.05**	1.40	0.28	1.12	0.35		
N_{min} soil	0.00	1.00	0.09	0.77	4.97	0.09	**12.57**	**0.01**

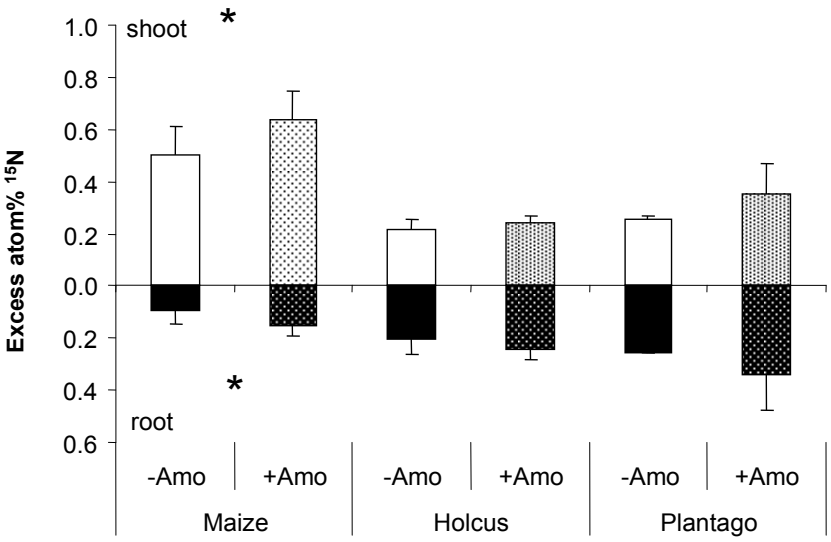

Figure 6. Excess atom%^{15}N in leaves (white bars) and roots (black bars) of *Zea mays* (Maize), *Holcus lanatus* (Holcus) and *Plantago lanceolata* (Plantago) in the presence (+Amo, dotted bars) and absence (-Amo, clear bars) of *Acanthamoeba castellanii*. Means+1SD; * p≤0.05

II.4. Discussion

The established microcosms system allowed evaluating plant growth and resource acquisition as affected by the microbial loop. Generally, densities of protozoa were comparable to other experiments (Griffiths 1990, Clarholm *et al.* 2006). Numbers of protozoa were increased in the presence of plants (Zwart *et al.* 1994), but were not influenced by plant species (Griffiths *et al.* 1992, Saj 2008).

Mineral N content peaked in bare soil in the presence of amoebae. In the presence of plants, protozoa did not enhance mineral N content in soil. In the short growing period of our experimental conditions, plants acquired about 13% of litter-^{15}N, suggesting that pants were strong N sinks, depleting the protozoan re-mobilized mineral N pool in soil. In agreement with our hypothesis plant species differed in the uptake of ^{15}N from the added litter. However, the N uptake from added litter, growth and morphological parameters of *H. lanatus* were not affected by the presence of protozoa. Thus, this species is either independent from protozoa for the acquisition of N from litter, or interacts with other symbionts, such as AM fungi, to increase nutrient uptake.

In the following, morphological and nutritional effects of amoeba on *Z. mays* and *P. lanceolata* are discussed in more detail.

Protozoa generally did not increase plant leaf and root biomass which is in agreement with e.g. Kuikman *et al.* (1990), but in contrast to most other published studies (Bonkowski 2004 and references therein). In agreement with our hypothesis protozoa induced species specific shifts in leaf and root surface of *Z. mays* and *P. lanceolata*.

The specific leaf area is an important parameter reflecting the favourable growth conditions e.g. concentrations of nutrients, especially N (Schulze *et al.* 2005). Furthermore, high specific leaf area indicates high metabolic activity (Schulze *et al.* 2005). Thus, even though leaf and root biomass were not affected, the increased specific leaf area indicates that *P. lanceolata* reacted to the presence of protozoa by enhancing its metabolism. This suggests that the presence of amoebae enhances plant fitness by creating more favourable growth conditions e.g. grazing induced shifts towards a more beneficial microbial community (Bonkowski and Brandt 2002). Additionally, greater specific leaf area correlates with enhanced rates of CO_2 assimilation (Schulze *et al.* 2005) which is the basis of C allocation into the rhizosphere. This suggests a morphological adaptation of *P. lanceolata* enabling the plant to stimulate microbial loop functioning *via* enhancing C exudation (Clarholm 1985, Bonkowski 2004, Lu *et al.* 2004).

As stated above, plant parameters showing a high standard-deviation that were possibly related to the plant transfer from axenic into more natural experimental conditions. Consequently we used specific root area as a parameter for root morphology, since it integrated root biomass and surface area. Additionally, the specific root area is the belowground analogue of the specific leaf area and reflects enhanced nutrient supply. Generally, root morphology varies with plant species (Kutchera and Lichtenegger 1982) which is directed by a genetic program (Zhang and Forde 1998), but the final configuration of the root system under natural conditions is largely determined by environmental factors (Zhang and Forde 1998, Hinsinger *et al.* 2005, Malamy 2005). Consequently, the increased specific root area in the presence of amoebae in *P. lanceolata* and *Z. mays* indicates favourable growth conditions probably due to enhanced nutrient availability (Clarholm 1985) or shifts in the microbial community composition (Kreuzer *et al.* 2006, Rosenberg 2008). This conclusion is supported by a number of studies reporting changes in root architecture of different plant species by protozoa (Jentschke *et al.* 1995, Bonkowski and Brandt 2002, Kreuzer *et al.* 2006). Shifts in root morphology is attributed to protozoan induced shifts in the rhizosphere bacterial community that enhances nutrient availability (Bonkowski and Brandt 2002, Kreuzer *et al.* 2006). However, enhanced nutrient availability could only be confirmed for *Z. mays* by an increased N uptake from added litter (^{15}N) in leaves and roots.

Plant roots are known to affect plant litter decomposition in soil (Van der Krift, Kuikman *et al.* 2001). The effects can either be positive, neutral or negative depending on the species of both litter and plant (Van der Krift, Gioachhini *et al.* 2001, Van der Krift,

Kuikman *et al.* 2001). *Zea mays* is considered to strongly modify the composition of the rhizosphere microbial community (Garbeva *et al.* 2004), resulting in increased N mobilization and uptake, indicated by ^{15}N. We suggest that *Z. mays* was better adapted to maximise N foraging *via* free living protozoa than *P. lanceolata* which might be due to the fact that the latter is highly dependent on the symbiosis with arbuscular mycorrhizal fungi (Gange and West 1994).

II.4.1. Conclusions

Conform to our hypothesis the studied plant species varied in their response to the presence of amoebae and therefore to the 'microbial loop'. This suggests close interactions between plant species and rhizosphere soil microbes in nutrient uptake from litter in soil. Both *Z. mays* and *P. lanceolata* are suitable model plants to investigate morphological and nutritional responses to the microbial loop, whereas *H. lanatus* did not respond to protozoan grazing on rhizosphere bacteria. The results illustrate that for studying rhizosphere interactions and their feedback to plants, plant species identity has to be considered.

We propose three research pathways to dissect the mechanisms in plant N uptake *via* the microbial loop in soil:

First, local or systemic effects on plant growth in N uptake and C partitioning induced by protozoan grazing need to be evaluated by using a "Split-Root" approach. In this approach, the root system can be manipulated on one side of the root system (e.g. treated with Amoeba) and systemic effects can be observed on the untreated part of the root system. *Zea mays* is an ideal model plant for this approach since it showed strong morphology plasticity and increased uptake of N from litter in soil in the presence of amoebae. Additionally, *Z. mays* has a big homorhizy root system, easily to divide and to transfer into the microcosms.

Second, other bacterial feeding fauna such as nematodes (Griffiths 1990) and potential interactions of amoebae with other symbionts that possess complementary function for plant N uptake from soil need to be studied. The most promising symbiont here are arbuscular mycorrhizal (AM) fungi that colonize 80% of all plant species (Smith and Read 1997). Arbuscular mycorrhizal fungi predominantly foster plant growth by enhancing mineral nutrient uptake from soil *via* an enhanced absorptive root area (Smith and Read 1997). In turn, AM fungi are obligate biotrophs receiving up to 30% of recently fixed photosynthates. *Plantago lanceolata* showed in our experiment strong morphological plasticity and is highly mycorrhizal (Gange and West 1994, Grime *et al.* 2007). Consequently, combining *P. lanceolata* with amoebae and AM fungi represents

an adequate model system to investigate multitrophic interactions in the acquisition of N and in turn the investment of C of the host plant in the symbionts.

Third, the role of litter quality for the functioning of the microbial loop needs to be investigated. Mineralization of N strongly varies with litter quality (Hodge *et al.* 2000). Consequently, the role of protozoa in providing N for plant uptake is likely to depend on litter quality and can be analysed by adding litter of different C-to-N ratio to the soil.

To dissect how multitrophic interactions in the rhizosphere impact the acquisition and partitioning of plant C, we will label plants with stable isotopes for tracing the fate of recently fixed C. Pulse labelling is a reliable method to follow C partitioning in the plant and allocation of C to rhizosphere symbionts (Todorovic *et al.* 2001, Henry *et al.* 2005, Robin 2006). Combining the use of litter labelled with ^{15}N with $^{13}CO_2$ pulse labelling of plants is expected to allow understanding the role of plant-protozoa interactions in plant N and C foraging. Additionally, stable isotope probing of phospholipid fatty acids may be used to identify shifts in metabolically-active rhizosphere microorganisms (Treonis *et al.* 2004) and their functions in N mobilization in the presence of amoebae (Lu *et al.* 2004).

Acknowledegments

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Chapter III. Effects of protozoa on plant nutrition and carbon allocation depends on the quality of litter resources in soil

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Summary

- By decomposing litter materials and mineralizing nutrients therein the decomposer system provides the basis for plant growth. Decomposition processes vary strongly with litter carbon-to-nitrogen (C-to-N) ratio and the mineralization of nutrients mainly relies on bacterial-feeding fauna, in particular amoebae.

- We assessed the effects of litter quality (C-to-N ratio) on the mobilisation of N and on plant growth (*Plantago lanceolata*) as modulated by grazing of protozoa (naked amoebae, *Acanthamoeba castellanii*) on rhizosphere bacteria ('microbial loop in soil'). ^{15}N labelled litter of low (C-to-N ratio 7, high quality litter, HQ) or high C-to-N ratio (C-to-N ratio 35, low quality litter, LQ) was added to microcosms to follow plant N uptake. Plant C partitioning was followed after pulse labelling of plant shoots with $^{13}CO_2$. Stable isotope probing (^{13}C) of phospholipid fatty acids (PLFAs) was used to identify shifts in the population of active rhizosphere microorganisms.

- Plant shoot and root biomass were lowest in the LQ treatment without amoebae and highest in the HQ treatment with amoebae. The proportion of total ^{15}N litter in plants at harvest was generally lower in the LQ than in the HQ treatment. Amoebae enhanced plant N uptake from litter and plant growth independent of litter quality. Plants allocated more recently fixed C to roots in the presence of amoebae but only in the LQ litter treatment. PLFAs were generally richer in ^{13}C in HQ litter as compared to LQ litter treatments. The presence of amoebae enhanced ^{13}C incorporation in PLFAs in LQ litter treatments only. Microbial community structure as indicated by PLFA profiles was predominantly affected by the quality of litter added to soil. Presence of amoebae altered microbial community structure in the LQ treatment only.

- Overall, the results suggest that *P. lanceolata* modulated the allocation of recently fixed C belowground in response to the microbial and nutritional environment in the rhizosphere. In presence of amoebae, N acquisition from litter and growth were maximised highlighting the beneficial effects of protozoa on plant performance.

III.1. Introduction

In most terrestrial ecosystems 80-90% of plant primary production enters the soil system as above- and belowground dead plant material ("litter") (Bardgett 2005). By recycling litter and mineralizing the nutrients therein, the decomposer system provides the basis of soil fertility and nutrient supply to plants. In turn, plants allocate a variety of organic resources in form of energy-rich rhizodeposits into the rhizosphere that fuel the activity, growth and diversity of heterotrophic microorganisms (Grayston *et al.* 1998, Baudoin *et al.* 2003, Lu *et al.* 2004). Consequently, rhizosphere microorganisms are assumed to be generally not limited by C (Griffiths *et al.* 2007). For growing, rhizosphere microorganisms need to mobilize nutrients from soil organic matter, such as N (Robinson *et al.* 1989, Nguyen 2003). Consequently, microorganisms break-down litter for mobilizing N and incorporating it into microbial tissue. By grazing on rhizosphere bacteria, microfauna, in particular amoebae (Clarholm 1985, Bonkowski 2004) and nematodes (Griffiths 1994a, Ruess and Ferris 2003, Mao *et al.* 2006), mobilize N from bacterial biomass, thereby making it available for plant uptake (Kuikman and van Veen 1989, Bonkowski 2004, Mao *et al.* 2006). This mechanism is termed "microbial loop in soil" (Clarholm 1985). Thus, nutrient cycling in ecosystems is a function of the microbial-feeding fauna (Bonkowski *et al.* 2000, Hodge *et al.* 2001). In case of amoebae, more than one third of the N fixed in bacterial biomass is excreted as NH_4^+, and the amount of N mobilized by protozoa has been calculated to be responsible for 20-40% of the N mobilized in the field (Kuikman and van Veen 1989, Griffiths 1994b, Bonkowski 2004). Additionally, amoebae affect N mineralization by increasing microbial activity (Bonkowski 2004) and by altering microbial community composition (Rønn *et al.* 2002, Rosenberg 2004).

Another important determinant for N mineralization is the quality of above- and belowground plant residues (Hodge *et al.* 2000a). At high litter C-to-N ratio, microorganisms immobilize N by incorporating it into microbial biomass thereby depleting the soil mineral N pool. Consequently, in presence of litter of high C-to-N ratio microorganisms compete with plants for inorganic N (Kaye and Hart 1997, Hodge *et al.* 2000a, Cheng and Gershenson 2007). Hodge *et al.* (2000a) calculated the minimum litter C-to-N ratio resulting in the release of N for both fungi and bacteria to be below 12.5 (Figure 7). If the litter C-to-N ratio exceeds 30.3, N is assumed to be sequestered into microbial biomass (Hodge *et al.* 2000a) and bacteria might be limited in their growth by N availability. Here, selective feeding of protozoa may strongly shift bacterial community structure, since compensative re-growth of dominant grazed groups may be

limited. At intermediate litter C-to-N ratios fungi release N but bacteria still sequester it (Hodge *et al.* 2000a) (Figure 7).

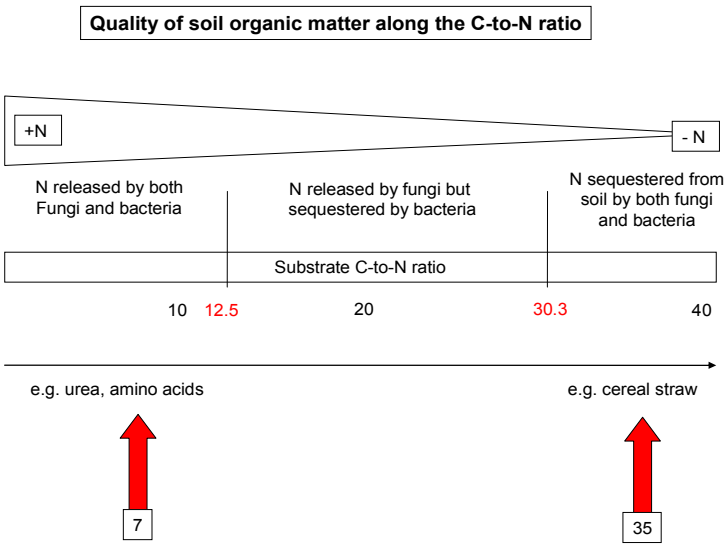

Figure 7. Relationship between the quality of soil organic matter, as indicated by its carbon-to-nitrogen ratio, and nitrogen mobilization/immobilization processes. Red arrows indicate the C-to-N ratio of the ^{15}N labelled litter used in our experiment. Red numbers indicate 'minimum' C-to-N ratios for the mobilization of nutrients from soil organic matter (adapted from Hodge et al. 2000).

We focused on the initial phase of litter decomposition since this phase is dominated by bacteria and their grazers (Freckman 1988, Griffiths 1990). Consequently, decomposition processes likely depend on litter C-to-N ratio of litter materials and bacterial feeders are likely to control N mobilization for plant uptake. This is the first study investigating variations in the mobilization of N by amoebae with litter quality.

In detail, the following hypotheses were investigated:

1. Amoebae increase N mobilization from bacterial biomass for plant uptake and growth with the effect being more pronounced in presence of low quality as compared to high quality litter;
2. Plant C allocation belowground and incorporation into microbial biomass is more pronounced in presence of low quality as compared to high quality litter;
3. Grazing by amoebae shifts the microbial community structure in particular in presence of low quality litter.

To prove these hypotheses we added either HQ (C-to-N ratio 7) or LQ litter (C-to-N ratio 35) to soil microcosms. A complex microbial community was established by re-inoculating sterilized soil with rhizosphere microorganisms and adding amoebae (*Acanthamoeba castellanii*) to the respective treatments. Plant (*Plantago lanceolata*) C allocation was studied by pulse labelling of shoots with $^{13}CO_2$. Mobilization of litter N was investigated by using ^{15}N labelled litter. Shifts in microbial community structure in

presence of amoebae were investigated by phospholipid fatty acid (PLFA) analysis in tandem with ^{13}C lipid stable isotopes probing.

III.2. Material and Methods

III.2.1. Microcosms

Soil was collected from the upper 20 cm from a grassland site grown on a former agricultural field, which had been abandoned for more than 10 years (van der Putten *et al.* 2000). The soil was stored in plastic bags at 4°C until use. To reduce nutrient concentrations the soil was mixed with sand at a ratio of 1:1. The soil-sand mixture was autoclaved (20 min, 121°C) and washed to deplete soil nutrients and toxic compounds. For chemical and physical properties of the soil-sand mixture see Table 4. Each treatment was replicated eight times, giving a total of 32 microcosms. To each microcosm (glass pots, 500 ml, Fisher Bioblock Scientific, Illkirch, France, Figure 8) fresh soil equivalent to 820 g dry weight was added and homogeneously mixed with either LQ or HQ litter (0.63 g; ground to powder; see below). Subsequently, microcosms with soil were autoclaved once again. Soil moisture content was adjusted to 75% of the water holding capacity. The bacterial inoculum[3] was prepared by subsequent filtering the supernatant of soil slurry though 5 µm and 1.2 µm filters (Bonkowski and Brandt 2002); 6 ml of the filtrate was added to each microcosm. Axenic *A. castellanii* were added to amoebae treatments after washing cultured amoebae in sterile filtered mineral water (3 ml; ca. 48000 individuals). Control treatments without amoebae received 3 ml of mineral water.

Table 4. Characteristics of washed soil-sand mixture (1:1) after the first autoclaving step [g kg^{-1}]

	soil-sand mixture (1:1)
Clay (< 2µm)	4
Silt fine (2-20 µm)	10
Silt coarse(20-50 µm)	9
Sand fine (50-200 µm)	89
Sand coarse (200-2000 µm)	888
Organic carbon	5.02
Total nitrogen	0.33
C-to-N ratio	15.3
Organic matter	8.68
pH	6.66
Phosphorus (P_2O_5)	0.07
Potassium (K_2O)	0.045
Potassium (K)	0.037

[3] Bacterial inoculum may have contained other soil organism than bacteria, e.g. spores of soil fungi

III.2.2. Plants and incubation conditions

Seeds of *Plantago lanceolata* were surfaced sterilized (Hensel *et al.* 1990). Single seeds were incubated in 96-well microtiter plates in 100 µl NB-NMAS for germination. The microtiter plates were incubated at 20°C in darkness and checked for microbial contaminations. Eight days old plants were transferred into sterile tubes filled with quartz sand and protected from contaminations by air borne cysts of amoebae by placement in a transparent glass container. Plants were aseptically transferred to microcosm after five days. Plants were grown in a climate chamber at 18 / 22°C night / day temperature, 70% humidity, 16 h photoperiod at 460 ± 80 µmol m^{-2} s^{-1} light photon flux density in the PAR range. Soil moisture was checked gravimetrically and kept at 75 % of the field capacity by adding of sterile distilled water every second day.

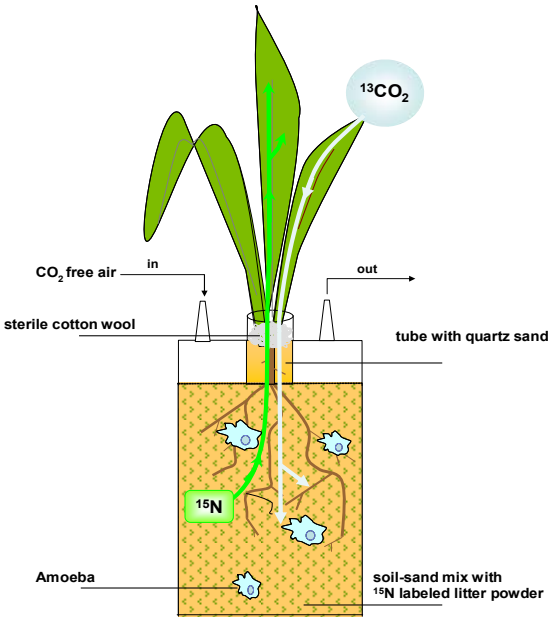

Figure 8. Microcosm set up. Plants (*Plantago lanceolata*) were grown in a soil-sand mixture (1:1) inoculated with a protozoa-free natural microbial community. Amoebae treatment contained axenic *Acanthamoeba castellanii* as microfaunal grazer. ^{15}N labelled *Lolium perenne* litter was homogeneously added to the soil to follow nitrogen uptake from soil. Plants were pulse labelled with $^{13}CO_2$ at the end of the experiment to follow plant C partitioning and transfer to below ground.

III.2.3. Preparation of ^{15}N labelled plant litter

Lolium perenne was grown in climate chamber at on a soil-vermiculite mixture in a growth chamber at 18 / 22°C night / day temperature, 70% humidity, 16 h photoperiod at 460 ± 80 µmol m^{-2} s^{-1} light photon flux density in the PAR range and watered with deionised water for 10 days. Then, shoots were cut at soil surface and discarded. To obtain shoot litter of contrasting C-to-N ratio plants were allowed to re-grow after adding $^{15}NH_4{}^{15}NO_3$ (10 atom%) at concentrations of 0.1 and 1.5 g l^{-1} to trays with plants for the production of LQ and HQ litter, respectively. Plants were cut again after 24 day and harvested after 10 weeks. Shoots were dried for 48 h at 80°C, ground and analysed for C, N and ^{15}N content (see below). The shoot litter C-to-N ratios (Table 2) fitted well to the contrasting litter quality categories as suggested by Hodge et al. (2000) (Figure 7). Litter N was sufficiently enriched in ^{15}N to be used as tracer for plant N uptake (Table 5).

Table 5. Percentages of carbon (C) and nitrogen (N), C-to-N ratio and atom%^{15}N of *Lolium perenne* litter of low quality (LQ) and high quality (HQ).

Litter	% C	% N	C-to-N ratio	atom%^{15}N
LQ	40.01	1.09	35	1.79
HQ	32.44	4.51	7	5.18

III.2.4. $^{13}CO_2$ pulse labelling of plants

Three weeks after transplanting the plants, microcosms were transferred into an assimilation chamber for subsequent pulse labelling with $^{13}CO_2$ (Robin 2007). Climatic conditions during the labelling period were same as those in the plant growth chamber (see above). First CO_2 concentration in the chamber was reduced rapidly by 50 % (10 min) to 180 vpm by passing the incoming air through a soda lime cartridge. Then, CO_2 partial pressure was re-adjusted rapidly to 360 vpm by addition of $^{13}CO_2$ generated by addition of 1 M lactic acid to $NaH^{13}CO_3$ (99 atom%). During the 5 h labelling period, CO_2 concentration in the chamber was kept at 360 vpm to compensate for plant assimilation with a mixture of $NaHCO_3$ at 50 atom%^{13}C and measured by an Infra Red Gas Analyser (IRGA; ADC 225 MK3, Hoddesdon, United Kingdom). Belowground respiration was measured during the first 48 h after labelling by passing CO_2 free air through the microcosms (n=4 per treatment) and into a 60 ml NaOH (1 M) trap (air flow ca. 18 ml min^{-1}).

Total C concentration in NaOH was measured in a TOC analyser (TOC-VCSH CSH/CNS, Shimadzu, Champs-sur-Marne, France). The ^{13}C isotopic excess of the trap was determined after precipitation of carbonates in saturated $SrCl_2$ (Harris *et al.* 1997)

and centrifugation. The supernatant was decanted and the pellet was freeze-dried before ^{13}C analysis by an elemental analyser coupled with an isotope mass spectrometer (see below).

III.2.5. Plant and soil analyses

Plants were destructively harvested 48 h after labelling. After removal of roots from soil by handpicking, subsamples of soil were taken. One part of the soil samples was dried (80°C, 48 h) and shoots freeze dried for biomass determination and the analysis of carbon and nitrogen concentrations by an elemental analyser (Carlo Erba, Na 1500 type II, Milan, Italy) coupled with an isotope mass spectrometer (Finnigan Delta S, Bremen, Germany).

III.2.6. Analysis of the $^{13}C/^{12}C$ and $^{14}N/^{15}N$ ratios of soil and plant samples

Samples were analyzed for ^{13}C and ^{15}N isotopes using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (IRMS, Sercon Ltd., Cheshire, UK). Samples were combusted at 1020°C in a reactor packed with chromium oxide and silvered colbatous/cobaltic oxide. Following combustion, oxides were removed in a reduction reactor (reduced copper at 650°C) and the helium carrier was passed through a water trap (magnesium perchlorate). Nitrogen and CO_2 were separated on a Carbosieve GC column (65°C, 65 mL min^{-1}) before entering the IRMS. Final delta ^{15}N and ^{13}C values were calculated by adjusting the provisional values such that correct values for laboratory standards were obtained. Standards were analyzed every 12th samples.

Data are presented in ^{15}N and ^{13}C in excess of the natural abundance. For ^{15}N and ^{13}C we used natural abundance (A_N) in plant tissue and soil samples of control plants grown at the same conditions as labelled plants (^{15}N in shoots and roots of 0.370 and 0.376‰, respectively; ^{13}C in shoots and roots of 1.076 and 1.079‰, respectively).

The amount of N and ^{15}N (mg) of a given organ or soil compartment were calculated as follows:

(1) $N_{tot} = BM_{sample} \times \left(\frac{\%N_{sample}}{100} \right)$; [where *BM* = Biomass of the considered plant or soil compartment]

(2) $^{15}N_{total} = N_{total} \times \left(\frac{atom\%^{15}N}{100} \right)$

For calculating the amount of ^{13}C we used delta values given by the mass spectrometer with belemnite as international standard.

The $\delta^{13}C$ values are defined as

(3) $\delta^{13}C = \left(\frac{R_{Sample}}{R_{Standard}} - 1 \right) * 1000;$

with R the ratio of stable isotopes

(4) $R_{Sample} = \frac{^{13}C}{^{12}C}$, $R_{Standard}$ = 0.01118 (VPDB);

Atom% ^{13}C defined as

(5) Atom% ^{13}C = 100 x F;

with F the fraction of the heavy isotope:

(6) $^{13C}F = \frac{(\delta + 1000)}{\left[\delta + 1000 + \left(\frac{1000}{R_{S\,\tan dard}} \right) \right]}$, according to Frey (2006).

III.2.7. PLFA patterns and lipid stable isotope probing

Analysis of PLFA patterns and $^{13}C/^{12}C$ ratio of individual PLFAs were used to monitor changes in the structure of the microbial community and to analyze active soil microbial populations. PLFAs were extracted from soil according to Frostegård *et al.* (1993) and subjected to a mild alcalic methanolysis to obtain fatty acid methyl esters (FAMEs). For more detail see Chapter 4 of this thesis.

Total PLFA was determined as the sum of all identified PLFA concentrations (nmol g^{-1}) (Zelles 1999). The quantities of the fatty acids (FAs) were obtained using 19:0 as the internal standard. The PLFAs chosen to identify bacteria were i15:0, a15:0, i16:0. 16:1ω7, cy17:0, and cy19:0, while 18:2ω6,9 was used to indicate saprotrophic fungi (Frostegård and Bååth 1996, Zelles 1999).

A gas-chromatography-combustion-isotope ratio-monitoring-mass spectrometer (GC-C-IRM-MS) system was used to determine the isotopic composition of individual FAs in soil samples. The system consisted of a gas chromatograph (6890 Series, Agilent Technology, USA) coupled via a Conflow II interface (ThermoFinnigan, Germany) to a MAT 252 mass spectrometer (ThermoFinnigan, Germany). A polar capillary column (FAME select, 50 m, 0.25 mm i.d., film thickness 0.25 mm) was used for the separation of FAMEs. The polar column was chosen due to its better separation of unsaturated

FAs compared to an unpolar column. For soil samples the GC split/splitless injector temperature was held at 250°C. The split flow was 1:3 and helium was used as carrier gas. The temperature program was set as follows: 60°C, 2 min isotherm, 20°C min^{-1} to140°C; 2°C min^{-1} to 160°C, 5 min isotherm, 2°C min^{-1} to 200°C, 10°C min^{-1} to 230 °C and held for 10 min. All samples were measured in at least three analytical replicates.

The carbon isotope composition is reported in δ notation (‰) relative to Vienna Pee Dee Belemnite standard (V-PDB) according to formula (3).The measured isotope ratios of the FAMEs were corrected for the isotope ratio of the methyl moiety to obtain the isotope ratios of the fatty acids. This was done by using the formula:

$$(7)\ \delta^{13}C_{FA} = C_n \frac{[(C_n + 1) \times \partial^{13}C_{FAME} - \partial^{13}C_{MeOH}]}{C_n}$$

where,

$\delta^{13}C_{FA}$ is the $\delta^{13}C$ of the fatty acid,

C_n is the number of carbons in the fatty acid,

$\delta^{13}C_{FAME}$ is the $\delta^{13}C$ of the fatty acid methyl ester (Abraham *et al.* 1998)

and $\delta^{13}C_{MeOH}$ is the $\delta^{13}C$ of the methanol (-38,83‰) used for the methylation reaction.

III.2.8. Counting of amoebae

Total numbers of amoebae were enumerated by the most probable number technique (Darbyshire *et al.* 1974). Here, 5 g of soil were dispersed in 20 ml NMAS and shaken for 20 min at 75 rpm. Aliquots of 0.1 ml were added to microtiter plates and diluted two fold in 50 µl sterile NB-NMAS. Microtiter plates were incubated at 15°C. Numbers were calculated according to Hurley and Roscoe (1983).

III.2.9. Quantification of microbial N

Microbial biomass N was determined in root free soil using the chloroform fumigation extraction (CFE) method as described by Vance *et al.* (1987) using 6 g fresh weight soil samples. Non-fumigated samples were extracted with 50 ml 0.5 M K_2SO_4 at 130 rev min^{-1} for 1 h and filtered subsequently. Extracted samples were kept frozen until analysis. Samples for fumigation were fumigated with ethanol-free chloroform for 24 h. Fumigated samples were extracted with 0.5 M K_2SO_4 as described for non-fumigated samples. A subsample was taken for analysis of N concentration in the K_2SO_4 extracts and measured in a TOC analyser (TOC-VCSH CSH/CNS, Shimadzu, Champs-sur-Marne, France) connected online to a N analyser (TNM-1, Shimadzu). Microbial biomass N was calculated as

(8) $N_{mic}CFE = \frac{¼E_n}{k_{en}}$

with E_n the difference between organic N extracted from fumigated and that extracted from non-fumigated samples, and k_{en} the efficiency constant 0.54 (Brookes *et al.* 1985). The other subsample was freeze dried and analysed for ^{15}N content. The percentage of ^{15}N in plants and microorganisms taken up from ^{15}N labelled litter added to soil was calculated as

(9) $\frac{^{15}N_{compartment}}{^{15}N_{litter}} * 100$

with compartment being either plants or microorganisms.

III.2.10. Statistical analyses

The effect of litter of different C-to-N ratio on the mobilization of N by amoebae for plant uptake were analysed by a full-factorial General Linear Model (GLM) with litter quality (HQ/LQ) and amoebae (without/with) as factors. For multivariate analysis of variance (MANOVA) the data set was divided into the following two coherent categories:

“plant” with shoot biomass, root biomass, plant biomass and shoot-to-root ratio and “mineral nutrients” with plant C-to-N ratio, percent uptake of ^{15}N from total ^{15}N in added to soil and C-to-N ratio of plant tissue.

Significant effects of the MANOVA were followed by separate factorial ANOVAs. Normal distribution and homogeneity of variance were improved by log-transformation (log[x + 1]). Data on percentages of total ^{15}N incorporated in plants and microorganisms were arcus sinus square root transformed (Sokal and Rohlf 1995). Differences in the overall structure of PLFA profiles between treatments were analysed by discriminant function analysis (DFA). DFA was performed in STATISTIKA 7 (Statsoft, Tulsa, USA), the other analyses were conducted in SAS 8.0 (Statistical Analysis System, SAS Institute Inc., USA).

III.3. Results

The model microcosm system successfully protected protozoa contaminations from airborne cysts; no protozoan contaminations were found in the treatments. Amoebal numbers ranged between 3332 ± 5378 per g dw soil in HQ litter treatments and 1662 ± 1546 per g dw soil^{-1} in LQ litter treatments.

III.3.1. Plant biomass, total C and N

Shoot and root biomass of *P. lanceolata* were lower in LQ litter as compared to HQ litter treatments (Figure 9).

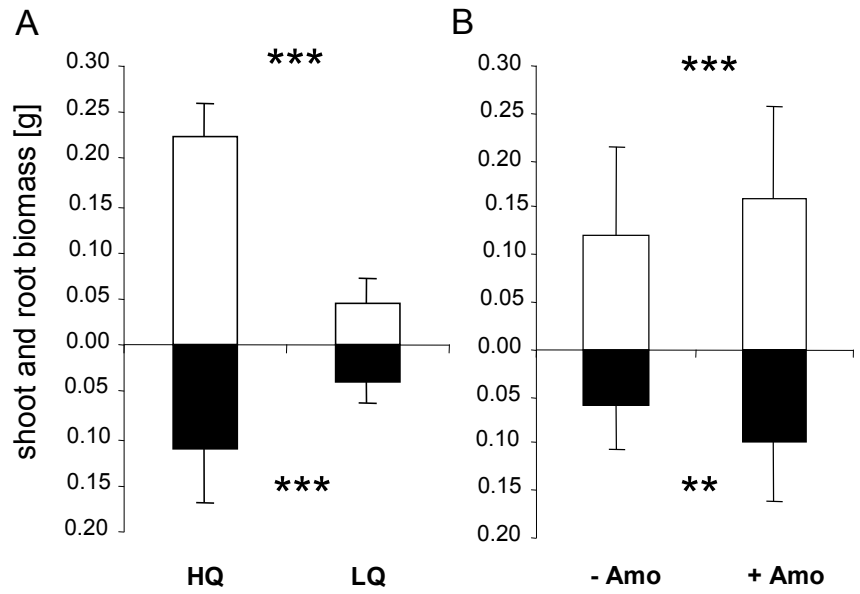

Figure 9. (A) Effects of high (HQ; C-to-N ratio 7) and low quality litter (LQ; C-to-N ratio 35) added to soil and **(B)** the presence (+AMO) and absence (-AMO) of amoebae on shoot and root biomass of *Plantago lanceolata*. Means + 1SD; *p≤0.05; ** p≤0.01, *** p ≤0.001).

Presence of amoebae increased plant shoot and root biomass irrespective of the quality of the litter added (Figure 9, Table 6). Consequently, plant biomass was at a maximum in HQ litter with amoebae and at a minimum in LQ litter without amoebae.

Table 6. F- and p-values of a two-factorial ANOVA on the effects of litter quality (C-to-N ratio) added to soil and the presence of amoebae on biomass [g], shoot-to-root ratio and C-to-N ratio of *Plantago lanceolata*

	Plant biomass				Shoot-to-root ratio		C-to-N ratio	
	shoot		root					
	$F_{1,24}$	p	$F_{1,24}$	p	$F_{1,24}$	p	$F_{1,21}$	p
Litter quality	287.95	0.0001	21.65	0.0001	37.89	<.0001	56.57	<.0001
Amoebae	17.53	0.0003	7.75	0.0103	0.29	0.597	0.97	0.3358
Litter quality x Amoebae	0.04	0.8389	1.4	0.2481	12.14	0.0019	4.63	0.0427

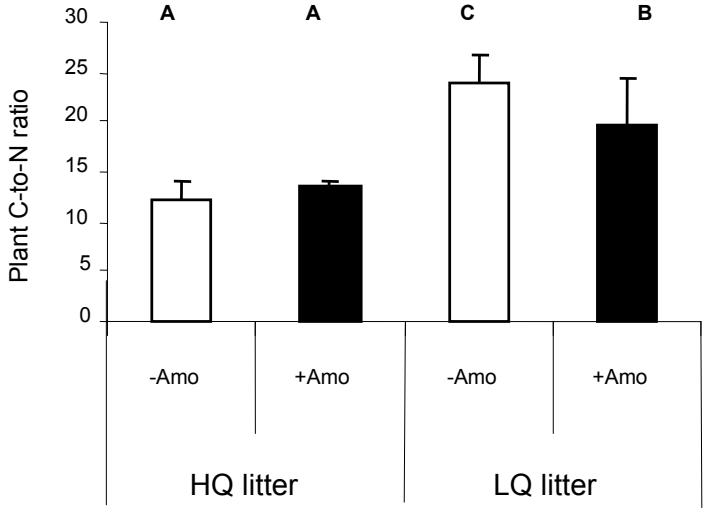

Figure 10. Effects of high (HQ; C-to-N ratio 7) and low quality litter (LQ; C-to-N ratio 35) added to soil on plant C-to-N ratio, in the presence (+AMO) or absence of amoebae (-AMO). Means + SD. Bars with the same letter are not significantly different (Tukey's HSD test; p≤0.05).

The effect of litter quality on tissue C-to-N ratio of *P. lanceolata* depended on the presence of amoebae (Figure 10, Table 6). Generally, C-to-N ratio in plants increased in LQ litter treatments by reducing total plant N (Table 7). Plant C-to-N ratio was at a maximum in LQ litter treatments in the absence of amoebae but decreased by a factor of 0.83 in their presence. This is related to a twofold increase of total N content in the presence of amoebae in LQ litter treatment (Table 7).

Table 7. Effects of high (HQ; C-to-N ratio 7) and low quality litter (LQ; C-to-N ratio 35) added to soil on total N and C in *Plantago lanceolata* in the presence (+AMO) or absence of amoebae (-AMO).

	HQ		**LQ**	
	-Amo	+Amo	-Amo	+Amo
N_{tot} [mg]	8.36±0.991	9.38±0.278	0.93±0.753	1.85±0.758
C_{tot} [mg]	102.9±24.63	126.9±6.44	20.9±15.33	36.4±14.87

Contrastingly, plant C-to-N ratio remained unaffected by the presence of amoebae in HQ litter treatment (Figure 10, Table 6). Here, the presence of amoebae increased the amount of total C and N in plant tissue by about the same factor and consequently plant C-to-N ratio remained unaffected (Table 6).

III.3.2. Plant and microbial ^{15}N and ^{13}C enrichment

Plant ^{15}N uptake from litter added to soil varied with litter quality (data not shown, Table 8). Shoot ^{15}N uptake from the added litter decreased from 13% in HQ to 0.34% atom%^{15}N in LQ litter treatments (data not shown, Table 8). The amount of ^{15}N in roots was lower than that in shoots. In parallel with shoots, ^{15}N uptake from the litter decreased from 2.4% in HQ to 0.29% in LQ litter treatments (data not shown, Table 8).

Amoebae only slightly affected the percentage of plant ^{15}N uptake from total ^{15}N added to soil but enhanced the amount of ^{15}N in shoots from 7.0 to 7.3% and that in roots from 1.4 to 1.6 % (Table 8). In HQ litter treatments the amount of ^{15}N in plants exceeded that in microbes (14.7-16.0 and 4.4-6.0 % of total ^{15}N added to soil, respectively). Conversely, in LQ litter treatments microorganisms captured more ^{15}N from the added litter than plants (6.3-9.1 and 0.7-0.9% of total ^{15}N, respectively).

III.3.3. ^{13}C enrichment of plant organs

Shoots and roots were generally more ^{13}C enriched in HQ as compared to LQ litter treatments (Figure 11, Table 8). The presence of amoebae affected atom%^{13}C only in the LQ treatment where they significantly increased ^{13}C allocation to roots by a factor of 1.12 (Figure 11, Table 8). Overall, plants allocated 24-27% of total fixed ^{13}C to roots in HQ litter treatments but 39-42% in LQ litter treatments.

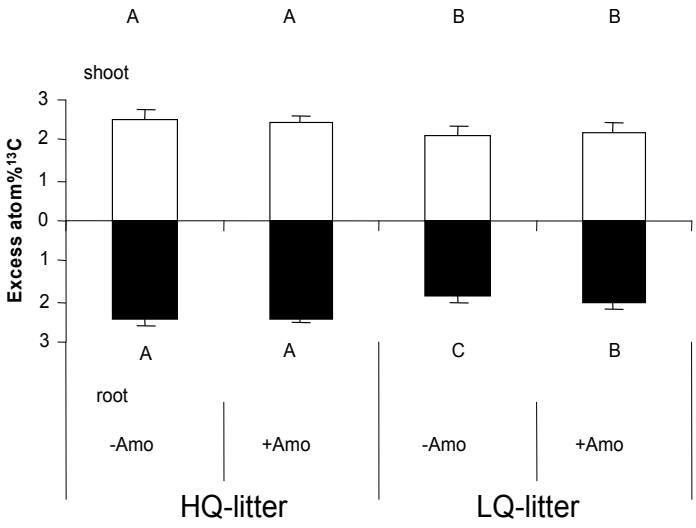

Figure 11. Effects of high (HQ; C-to-N ratio 7) and low quality litter (LQ; C-to-N ratio 35) added to soil on atom% ^{13}C in shoots and roots, in the presence (+AMO) or absence (-AMO) of amoebae. Means + 1 SD. Bars with the same letter are not significant different (Tukey's HSD test; p≤0.05)

III.3.4. ^{13}C enrichment of belowground respiration

The total ^{13}C in belowground respiration was not affected by litter quality but almost doubled in LQ litter treatments in the presence of amoebae (Figure 12, Table 8). The total belowground respiration followed the same pattern (data not shown).

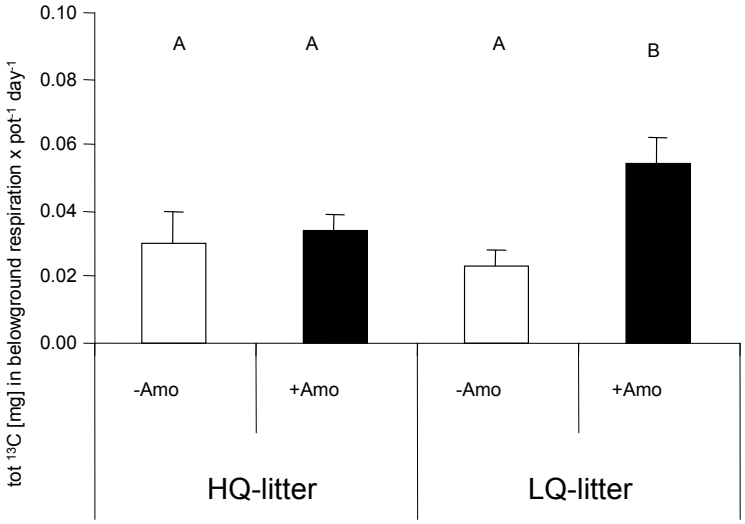

Figure 12. Effects of high (HQ; C-to-N ratio 7) and low quality litter (LQ; C-to-N ratio 35) added to soil on total ^{13}C content in belowground respiration (expressed per microcosm per day) in presence (+AMO) or absence (-AMO) of amoebae. Means + 1 SD. Bars with the same letter are not significantly different (Tukey's HSD test; p≤0.05)

In total, 13 predominant PLFAs with a chain length between 14 and 19 carbon atoms were detected. Total amount of microbial (overall mean 16.6 ± 3.6 nmol g^{-1}) and

bacterial PLFAs (overall mean 6.3 ± 1.2 nmol g^{-1}) did not differ between litter treatments ($F_{1,23}$ = 0.71, p = 0.41 and $F_{1,23}$ = 2.58, p = 0.12, respectively).

Table 8. F- and p-values values of a two-factorial ANOVA on the effects of litter quality (C-to-N ratio) added to soil and the presence of amoebae on ^{15}N uptake of *Plantago lanceolata* from added litter, atom%^{13}C in shoots and roots, and total ^{13}C in belowground respiration.

	^{15}N uptake from labelled litter				**atom%^{13}C**				**total ^{13}C in belowground respiration**	
	shoot		Root		shoot		Root			
	$F_{1,21}$	p	$F_{1,21}$	p	$F_{1,21}$	p	$F_{1,21}$	P	$F_{1,11}$	p
Litter quality	831.24	<0.0001	284.82	<0.0001	15.19	0.0008	81.76	<0.0001	3.7101	0.0803
Amoebae	4.22	0.052	4.96	0.037	<0.01	0.9828	3.43	0.0774	24.262	0.0004
Litter quality x Amoebae	2.7	0.1153	0.57	0.459	1.86	0.1865	5.36	0.0303	15.347	0.00239

III.3.5. Phospholipid fatty acids

In contrast to the total amount of microbial PLFAs, microbial community structure, as indicated by PLFA patterns, varied significantly with litter quality. Amoebae modified PLFA patterns only in LQ but not in HQ litter treatment (Figure 13).

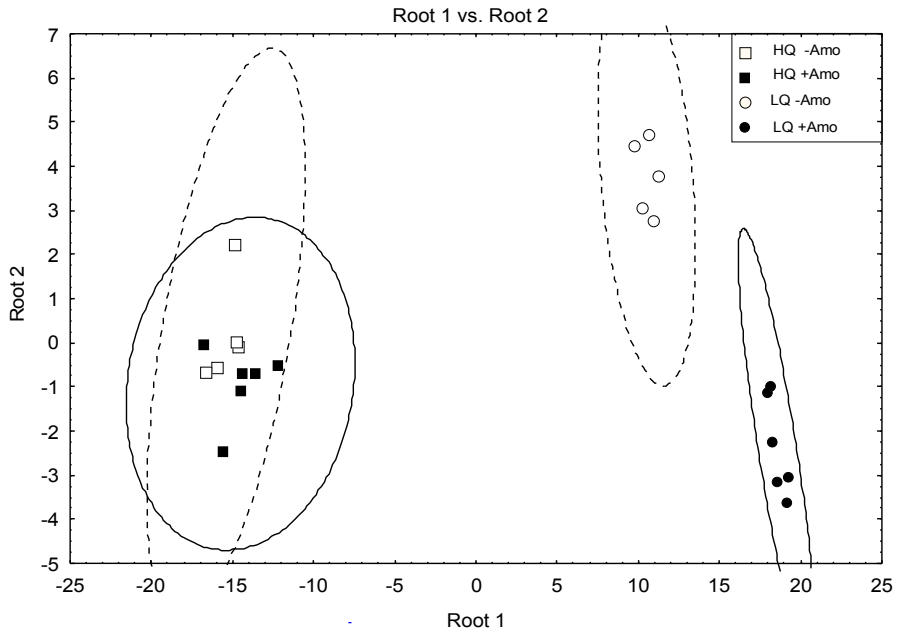

Figure 13. Discriminant function analysis of the effect of high (HQ, C-to-N ratio 7) and low quality litter (LQ, C-to-N ratio 35) added to soil and the presence (+AMO) and absence (-AMO) of amoebae on the composition of phospholipid fatty acids as indicators of microbial community composition (Wilks' Lambda = 0.0029, $F_{39,18}$ = 9.11, p < 0.001, eigenvalues of 281.45 and 6.10 for root 1 and root 2, respectively). Ellipses represent confidence ranges at α = 0.05.

Fungal (18:2ω6), $Gram^+$ (i16:0) and general bacterial PLFA (16:1ω7) and unspecific PLFA (18:0) correlated negatively with canonical scores of the first root, whereas $Gram^-$ (cy17:0) and the bacterial 18:1ω9t PLFA correlated positively with the second root (Table 9). Further, the PLFA 16:1ω5, reflecting predominantly arbuscular

mycorrhizal fungi, contributed to discriminate litter quality treatments along the first and amoebal treatments along the second axis (Table 9).

Table 9. Correlation between canonical scores of the first and second root with concentrations of individual phospholipid fatty acids. Pearson correlation coefficients; significant correlations are indicated by* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

	Root 1	**Root 2**
14:0	-0.3635	0.2122
i15:0	-0.0864	0.228
a15:0	-0.3598	0.1871
i16:0	-0.6545***	0.0584
16:1ω7	-0.5403**	0.1931
16:1ω5	0.4456*	0.6089**
16:0	0.1511	0.195
cy17:0	0.234	0.5275*
18:2ω6	-0.6573***	-0.0323
18:1ω9	0.2279	0.3305
18:1ω9t	0.0286	0.5308*
18:0	-0.6716***	0.2563
cy19:0	-0.3354	0.3296

III.3.6. $\delta^{13}C$ signatures of PLFAs

Discriminant function analysis of $\delta^{13}C$ signatures of PLFAs showed a similar pattern as total PLFAs (eigenvalues of 55.9 and 3.47 for roots 1 and root 2, respectively, Wilks' Lambda = 0.0024; $F_{27,21} = 5.36$, $p < .0001$, Figure 14).

In general, PLFAs were relatively more enriched in ^{13}C in HQ as compared to LQ litter treatments. 18:1ω9c, present in prokaryotes and in eukaryotes, was enriched at the same level as 16:0 and showed strong standard deviation possibly due to remaining ^{13}C enriched plant residues in extracted soil samples that contained low amounts of the latter PLFAs (Figure 15).

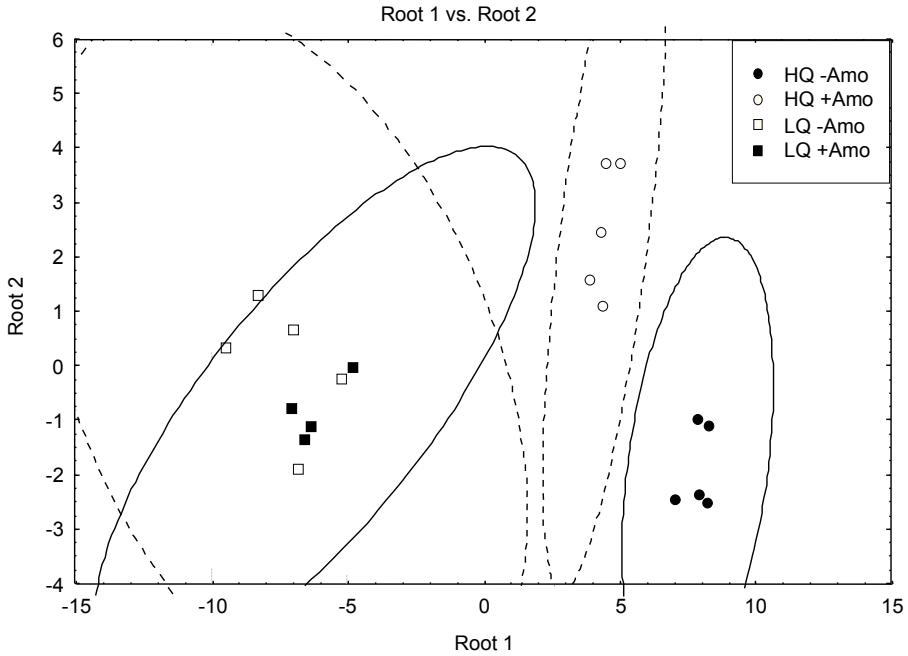

Figure 14. Discriminant function analysis of the effect of high (HQ, C-to-N ratio 7) and low quality litter (LQ, C-to-N ratio 35) added to soil and the presence (+AMO) and absence (-AMO) of amoebae on $^{13}C/^{12}C$ ratios in the phospholipid fatty acids (Wilks' Lambda = 0.0024; $F_{27,21} = 5.36$, $p < 0.0001$, eigenvalues of 55.9 and 3.47 for roots 1 and root 2, respectively). Ellipses represent confidence ranges at $\alpha = 0.05$.

Especially bacterial 14:0 (predominantly as short chain in bacterial microsoms) and 16:1ω7 (predominantly gram$^-$ bacteria) were relatively more enriched in ^{13}C in HQ as compared to LQ litter treatments. In HQ treatments, 14:0, i15:0, a15:0 and 18:2ω6 showing the same enrichment in HQ litter treatments with delta ^{13}C value of -16 (Figure 15). The PLFA cy17:0 was less enriched in HQ treatments with delta ^{13}C value of -20.0. In the presence of amoebae ^{13}C incorporation into PLFAs increased generally in HQ treatments, except for 16:1ω7 and 18:1ω9 (Figure 15).

In LQ litter treatments ^{13}C incorporation generally increased in the presence of amoebae but remained unaffected for a15:0 and cy17:0 (Figure 14, Figure 15). PLFA cy17:0 showing the highest increase of ^{13}C incorporation in the presence of amoebae (Figure 15).

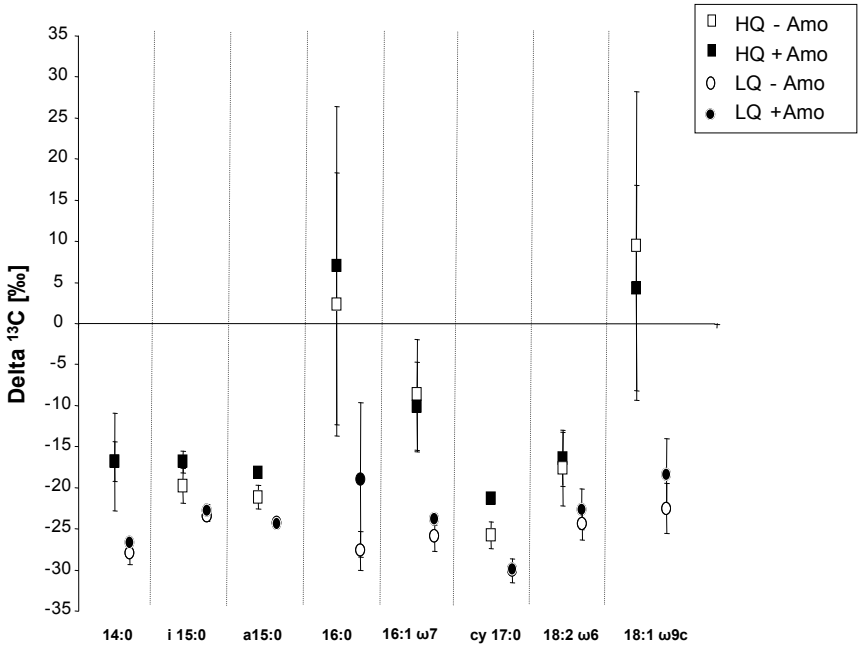

Figure 15. Effects of high (HQ; C-to-N ratio 7) and low quality litter (LQ; C-to-N ratio 35) added to soil on the incorporation of recently fixed C ($\delta^{13}C$) into PLFAs in presence (+AMO) or absence (-AMO) of amoebae.

III.4. Discussion

For the first time, this study integrated litter quality as driving factor of multitrophic rhizosphere interactions. The established model system combined with stable isotope labelling allowed to quantitatively investigate effects of multitrophic interactions on nutrient acquisition and C allocation of plants. As expected, amoebae in the rhizosphere of plants fostered plant N uptake. However, in contrast to our expectation the effects were insensitive to litter quality. Further, amoebae shifted microbial community structure but only in presence of low quality litter. Consistent with the model of Hodge *et al.* (2000a), in presence of HQ litter, N was mobilized for plant uptake rather than sequestered by microorganisms. In contrast, in LQ litter treatments N was sequestered into microbial biomass suggesting that they efficiently competed with plants for nutrients. As a consequence, and consistent with earlier experiments, plants took up more litter N in HQ as compared to LQ litter treatments, suggesting reduced success for nutrient competition with microorganisms in LQ treatments (Kaye and Hart 1997, Hodge *et al.* 2000a, Hodge *et al.* 2000b, Hättenschwiler *et al.* 2005, Wardle *et al.*

2006). Subsequently, plant growth was reduced and the plant tissue produced was poor in N (high C-to-N ratio).

Amoebae stimulated plant N acquisition from the added litter even in presence of low quality litter. The results suggests that in each of the treatments the C-to-N ratio of bacteria as compared to protozoa remained low and therefore protozoa continuously excreted excess N. Parallel to the effect of amoebae on litter nutrient mobilization, plants responded to the presence of amoebae by changing C allocation patterns. However, this was restricted to the LQ litter treatment where in agreement with our second hypothesis, plant increased C allocation belowground in the presence of amoebae. As a consequence of an effective ^{13}C fixation in HQ litter treatments, the concentration of ^{13}C in plant tissue was higher than in LQ litter treatments. Conversely, C partitioning into roots was strongest in LQ litter treatments, indicating that plant roots represented a strong C sink in these treatments. Furthermore, the amount of ^{13}C in belowground respiration peaked in LQ litter treatments in the presence of amoebae indicating enhanced transfer of recently fixed C into the rhizosphere and/or enhanced metabolic activity of roots and microbes.

To dissect how plant derived C is incorporated into microbial populations depending on the nutritional conditions and the presence of amoebae, we used compound specific ^{13}C PLFA stable isotope probing. Generally, PLFAs were more enriched in ^{13}C in HQ litter treatments and also in LQ treatments in the presence of amoebae. This suggests that amoebae in LQ litter treatments indeed enhanced the incorporation of ^{13}C into rhizosphere microorganisms and that at least in part the increase in the amount of ^{13}C respired was due to enhanced activity of rhizosphere microorganisms. This is in contrast to the HQ litter treatments where PLFAs were generally more enriched in ^{13}C but the amount of ^{13}C in soil respiration was lower as compared to LQ litter treatments with amoebae. This suggests that recently fixed photosynthates were locked up in microbial biomass and little used for energy metabolism. As indicated by PLFA analysis the growing microbial populations supplied with both plant C and litter N were little affected by grazing by amoebae, suggesting that growing microbial populations are little controlled by consumers. This is further supported by the fact that the pattern in enrichment in ^{13}C in PLFAs in HQ litter treatments was not affected by amoebae, suggesting that grazing by amoebae did not shift the use of plant C of microbial populations. However, the analysis generally indicates that rhizosphere microbial populations differ in the degree they incorporate plant derived C (Lu *et al.* 2004, Paterson *et al.* 2007). Further, low $\delta^{13}C$ values in certain PLFAs suggest that a number of rhizosphere microorganisms at least in the short-term do not incorporate root derived

resources. Even though isotopic signatures of PLFAs are closely related to environmental conditions, a number of microbial populations may exist in a dormant state with low C turnover (Kramer and Gleixner 2008). As indicated by $\delta^{13}C$ values in cy17:0 this may apply in particular to Gram$^-$ bacteria. Contrastingly, the bacterial PLFA 16:1ω7 were more enriched in ^{13}C in HQ as compared to LQ litter treatments suggesting that certain bacteria preferentially incorporated plant derived C. High enrichment in ^{13}C in 18:2ω6 further indicates that root derived C resources were also heavily used by saprophytic fungi.

Microbial community structure and incorporation of recently fixed photosynthates into PLFAs were predominantly affected by litter quality suggesting that microbial community structure was mainly controlled by the availability of litter N, i.e. was mainly bottom-up controlled by nutrients rather than plant derived C resources. This agrees with the widely held assumption that microorganisms in the rhizosphere of plants are limited by nutrients rather than C (Griffiths *et al.* 2007). Only in LQ litter treatments, microorganisms were also controlled by amoebal grazing, i.e. by top-down forces. Here, in particular Gram$^-$ bacteria separated grazed and ungrazed microbial communities. Indeed, it is known that amoebae preferentially graze on Gram$^-$ bacteria thereby stimulating their activity and turnover (Foster and Dormaar 1991, Andersen and Winding 2004). This is in accord with the view that amoebae induce shifts in microbial community structure towards gram$^-$ plant growth favouring populations (Bonkowski 2004), such as *Pseudomonas* species, which are among the most important and widespread plant growth promoting rhizobacteria (Lugtenberg *et al.* 2002). Pseudomonads control plant pathogens by e.g. producing antibiotics and inducing systemic resistance against eukaryotes in particular pathogenic fungi (van Loon *et al.* 1998, Pieterse *et al.* 2002, Bakker *et al.* 2007).

III.4.1. Conclusions

This study for the first time showed that plant C allocation and nutrient mobilization from litter resources of different quality depends on the presence of amoebae, i.e. on plant-bacteria-protozoa interactions. Plants adjusted the allocation of C resources to roots and into the rhizosphere depending on litter quality and the presence of bacterial grazers. This modified allocation pattern lead to an increased plant growth. Especially in LQ litter treatments, the mobilization of N resources by amoebae substantially increased plant performance by increasing leaf N concentrations thereby stimulating C assimilation. As a consequence, more C was allocated to roots and into the rhizosphere thereby stimulating microbial activity and the amoebae-mediated

mobilization of N and promotion of beneficial microbial rhizosphere communities. Thus, increased allocation of recently fixed photosynthates into the rhizosphere in the LQ litter treatment only fed back to the benefit of plants in the presence of amoebae. Conversely, in HQ treatments where N was easily accessible, plants and microbes used C resources mainly to build up biomass. However, if N was easily accessible the presence of amoebae also beneficially affected plant growth but as indicated by PLFA analysis this was based on increased availability of nutrients and not on changes in microbial community structure. This suggests that even if nutrients are easily accessible grazing on bacteria increase plant nutrient accessibility.

In natural ecosystems plants form associations with multiple root infecting and free living symbionts including AM fungi and rhizobia and live in association with other plants. Thus, plants are targets of diverse interactions, resulting in increased competition for resources (Grimoldi *et al.* 2005, Craine 2006) but also facilitative interactions which likely interact with each other and these interactions may be modulated by plants to increase fitness. To fully explore the role of rhizosphere symbionts multiple symbionts and their interactions need to be considered.

Acknowledgements

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Chapter IV. Protozoa (*Acanthamoeba castellanii*) and arbuscular mycorrhizal fungi (*Glomus intraradices*) mediate the partitioning of carbon and the availability of nitrogen for Plantago lanceolata

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Summary

- In most terrestrial ecosystems nitrogen (N) is the primary limiting nutrient for plant growth. To meet their need for mineral N, plant roots interact with multiple free living and root infecting symbionts of different trophic levels. However, most studies considered single plant mutualists only ignoring potential complementary interactions to the benefit of the host plant.

- The objective of our work was to investigate if AM fungi (*Glomus intraradices*) and protozoa (*Acanthamoeba castellanii*), both common and abundant root symbionts, complement each other in fostering N acquisition of plants (*Plantago lanceolata*) from decomposing litter in soil. Plants allocate C and stimulate activity and growth of heterotrophic plant symbionts. We also evaluated how different symbionts feed back on C partitioning in the plant and in the rhizosphere.

- In order to dissect interactions between plant, protozoa and AM fungi interactions in foraging of N and C, we added ^{15}N labelled litter into the soil and labelled shoots with a pulse of $^{13}CO_2$. Phospholipid fatty acid profiles were used to identify shifts in the population of rhizosphere microorganisms.

- Protozoa promoted plant growth by re-mobilizing N from fast growing rhizobacteria and by fostering microbial activity. They enhanced mineral N content in soil and shifted microbial community structure. AM fungi also contributed to plant N nutrition, most likely through enhancing the absorptive surface in soil and the transport of mobilized N to the host plant. AM fungi and protozoa complemented each other in an additive way and increased plant growth by maximising N uptake to foster carbon foraging and allocation to both symbionts.

- For optimizing plant nitrogen nutrition multiple mutualistic partners, including protozoa and AM fungi, are necessary. The separation of root and hyphal interactions is crucial to unravel the mechanisms in N uptake and C partitioning in the amoeba-mycorrhiza-plant-symbiosis.

IV.1. Introduction

Mineral nitrogen (N) limits plant growth in most ecosystems (Vitousek and Howarth 1991, LeBauer and Treseder 2008). To meet their need for mineral N, plant roots are associated with multiple root infecting and free living symbionts of very different phylogenetic affiliation and trophic levels (Marschner 1995, Phillips *et al.* 2003, Bonkowski 2004).

In terrestrial ecosystems, more than 80% of the flowering plants interact with root colonizing arbuscular mycorrhizal (AM) fungi (Smith and Read 1997). AM fungi are obligate biotrophs receiving up to 20% of recently fixed photoassimilates (Jakobsen and Rosendahl 1990, Bago 2000). Most of the beneficial effects of AM fungi on plant growth are attributed to nutrient uptake of poorly mobile nutrients, predominantly phosphorus (Smith and Read 1997). However recently, Hodge et al. (2001) showed that AM fungi also foster plant N nutrition by extending the absorptive surface and exploiting nutrients beyond the depletion zone of roots (Hodge *et al.* 2001, Govindarajulu *et al.* 2005).

In addition to root infecting symbionts (Lum and Hirsch 2003), plant roots interact with free living microorganisms in the rhizosphere. For example protozoa have been shown to stimulate microbial activity and mineralization of organic matter (Bonkowski *et al.* 2000) and release high amounts of N from consumed bacterial biomass into soil (Kuikman and van Veen 1989). Excreted N becomes available for plant and enhances plant growth. This beneficial effect of protozoa is commonly ascribed to “the microbial loop in soil” (Clarholm 1985, Coleman 1994). The microbial loop concept assumes that easily available C compounds released from roots trigger the mobilization of N from organic residues (“litter”) by bacteria. Bacterial grazers subsequently re-mobilize the nitrogen pool locked up in the bacterial biomass, rendering it available for plant uptake. Protozoa excrete one third of the ingested N as NH_4^+ resulting in an marked increase in plant growth (Griffiths 1994). Further protozoa affect N mineralization by increasing microbial activity (Bonkowski 2004) and altering microbial community composition (Rønn *et al.* 2002b). Additionally there is strong evidence that protozoa induce non-nutritional effects *via* increasing the biomass and activity of beneficial microorganisms, e.g. nitrifying or auxin producing bacteria (Bonkowski and Brandt 2002, Bonkowski 2004).

Despite the ubiquity of mycorrhiza and protozoa in rhizosphere soil and their importance for plant nutrition and growth, only few studies investigated their

interactions (Bonkowski *et al.* 2001, Wamberg *et al.* 2003, Herdler *et al.* 2008, Vestergard *et al.* 2008).

As indicated above AM fungi increased N uptake. Protozoa are known to stimulate microbial mineralizing and release of N from consumed bacteria. Therefore interactions of AM fungi and protozoa on plant N acquisition are likely.

The objectives of this experiment were to investigate (1) AM fungi and protozoa interactions in plant N uptake (2) C fixation and allocation, and (3) how this feeds back to microbial community structure.

We hypothesize that:

Arbuscular mycorrhizal fungi and protozoa interact to improve plant N uptake and growth by re-mobilizing mineral N from bacterial biomass.

Enhanced plant N uptake increases plant C assimilation and consequently stimulates C allocation to roots and into the rhizosphere thereby fostering symbiont functioning.

Protozoa shape microbial community structure by grazing on bacteria.

The addition of ^{15}N labelled substrates to follow N transfer into the plant has been applied for *P. lanceolata* (Chapter 2 this thesis and Hodge et al. 2001). We used $^{13}CO_2$ pulse labelling of aboveground plant parts to follow C allocation in the plant and transfer to the different microbial symbionts in the rhizosphere (Lu *et al.* 2004, Paterson *et al.* 2007). The extraction of phospholipid fatty acids (PLFA) from soil is a powerful method to separate microbial populations in soil microbial communities (Ramsey *et al.* 2006) and was applied to uncover shifts in microbial community structure (Frostegård *et al.* 1993a, Frostegård and Bååth 1996, Butler *et al.* 2003, Kirk *et al.* 2004). The method complements our approach investigating how plant N acquisition is mediated by AM fungi and microbial food web (protozoa) interactions.

IV.2. Material and Methods

IV.2.1. Microcosms, soil and microorganisms

Soil was collected from the upper 20 cm from a grassland site grown on a former agricultural field, which had been abandoned for more than 10 years (Van der Putten *et al.* 2000) and stored in plastic bags at 4°C until use. The soil was mixed at a ratio of 1:1 with sand (for more details see Table 10).

Table 10. Analysis of washed soil-sand mixture (1:1) after first autoclaving step [^{1}g kg^{-1}]

	soil-sand mixture (1:1)
Clay (< 2µm)	4
Silt fine (2-20 µm)	10
Silt coarse(20-50 µm)	9
Sand fine (50-200 µm)	89
Sand coarse (200-2000 µm)	888
Organic carbon	5.02
Total nitrogen	0.33
C-to-N ratio	15.3
Organic matter	8.68
pH	6.66
Phosphore (P_2O_5)	0.07
Potassium (K_2O)	0.045
Potassium (K)	0.037

The soil-sand mixture was autoclaved (20 min, 121°C) and washed with a threefold volume of tap water to deplete the soil on nutrients and toxic compounds mobilized by autoclaving. Milled ^{15}N labelled *L. perenne* litter (45.2 atom% ^{15}N, C-to-N ratio 15.1) was mixed with non-labelled *L. perenne* litter (C-to-N ratio 16.5) to obtain litter containing 10 atom% ^{15}N. From the litter 0.47 g was homogeneously mixed with 780 g soil (dry weight) and transferred into microcosms. The microcosms were again autoclaved and the soil moisture content adjusted to 75% of the water holding capacity.

A natural protozoa-free bacterial inoculum[4] was prepared by filtering the supernatant of a soil slurry through a filter of 5 µm mesh size, followed by a second filter of 1.2 µm mesh size (Bonkowski and Brandt 2002). Each microcosm was inoculated with 6 millilitres of the filtrate. The protozoa treatments received 2 ml of *A. castellanii* culture (ca. 95000 individuals equivalent to ca. 100 ind. g^{-1} soil) that has been washed in sterile filtered mineral water. Two ml of mineral water were added to non-protozoa treatments.

IV.2.2. Plant preparation and growth conditions

Seeds of *Plantago lanceolata* (Appels Wilde Samen GmbH, Darmstadt, Germany) were surfaced sterilized (Hensel *et al.* 1990) and separately germinated in 96-well microtiter plates filled with 100 µl sterile nutrient broth mixed with Neff's Modified Amoebae Saline (NB-NMAS) at 1:9 v:v (Bonkowski and Brandt 2002). Subsequently, the microtiter plates were incubated at 20°C in darkness and checked for microbial contaminations. Eight days after germination the plants were transferred into sterile

[4] Bacterial inoculum may have contained other soil organism than bacteria, e.g. spores of soil fungi

tubes filled with quartz sand. Plants of the mycorrhizal treatment were inoculated with agar pieces of approximately 4 mm³ containing spores and mycelium of the axenic AM fungi *Glomus intraradices,* SCHENK (Bago *et al.* 1996). After 5 days the tubes were transferred into the microcosms (Figure 16). Plants were grown in a growth chamber at 18°C / 22°C night/ day temperature, 70 % of humidity, 16 h of light at 460 ± 80 µmol m^{-2} s^{-1} light photon flux density in the PAR range at plant level. Soil moisture was maintained gravimetrically at 75 % of the field capacity every second day.

IV.2.3. $^{13}CO_2$ pulse labelling and quantification of ^{13}C respiration of the belowground compartment

Thirty days past transplantation of the seedlings, microcosms were transferred into an assimilation chamber for subsequent pulse labelling with of $^{13}CO_2$ (Robin 2007). Climatic conditions during the labelling period were the same as those in the plant growth chamber (see above). The first step of the labelling procedure was to reduce rapidly, CO_2 concentration in the chamber by 50 % (10 min) to 180 vpm by forcing the air to pass through a soda lime cartridge and subsequently CO_2 partial pressure rapidly re-adjusted to 360 vpm by addition of $^{13}CO_2$ generated by addition of 1 M lactic acid to $NaH^{13}CO_3$; (99 atom%). During the 5h labelling period, CO_2 concentration in the chamber was kept at 360 vpm to compensate for plant assimilation with a mixture of $NaHCO_3$ at 50 atom% ^{13}C and measured by an Infra Red Gas Analyser (IRGA; ADC 225 MK3, Hoddesdon, United Kingdom). Belowground respiration was measured during the first 48 h after labelling by passing CO_2 free air through the microcosm s(n=4 per treatment) and into a 60 ml NaOH (1M) trap (air flow ca. 18 ml /min^{-1}).

Total C concentration in NaOH was measured using a TOC analyser (TOC-VCSH CSH/CNS, Shimadzu, Champs-sur-Marne, France). The ^{13}C isotope excess was determined after precipitation of carbonates in saturated $SrCl_2$ (Harris *et al.* 1997) and centrifugation. The supernatant was decanted and the pellet was freeze-dried before ^{13}C analysis by an elemental analyser coupled with an isotope mass spectrometer (see below).

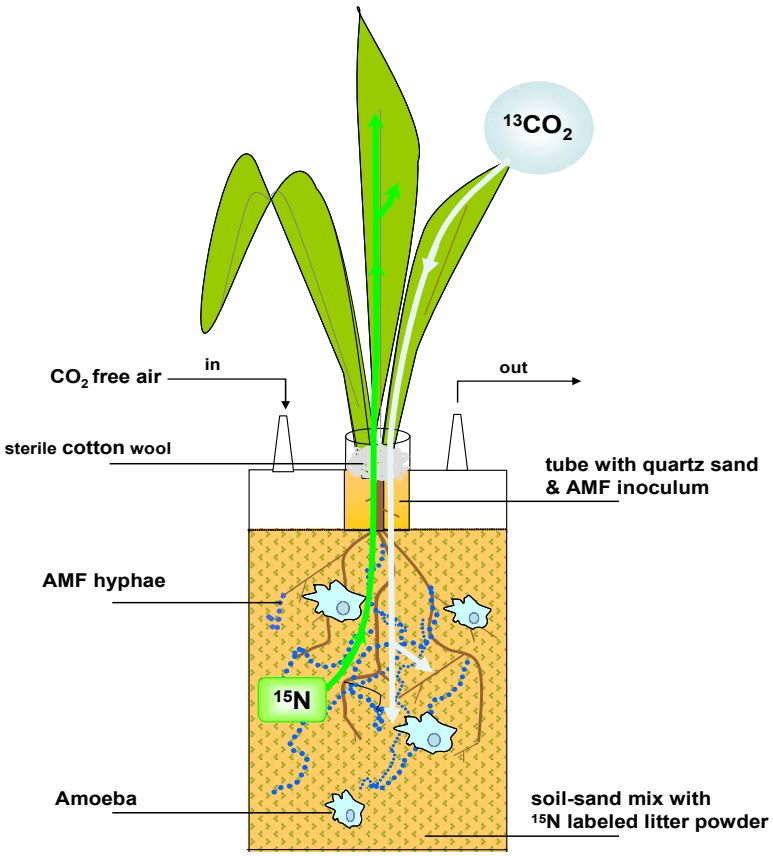

Figure 16. Microcosm set up. Plants (*Plantago lanceolata*) were inoculated with arbuscular mycorrhizal fungi (AMF; *Glomus intraradices*) and non mycorrhizal plants were grown in a soil-sand mixture (1:1) inoculated with a protozoa-free natural microbial community. Amoebae treatment contained axenic *Acanthamoeba castellanii* as microfaunal grazer. ^{15}N labelled *Lolium perenne* litter was homogeneously added to the soil to follow nitrogen uptake from soil. Plants were pulse labelled with $^{13}CO_2$ at the end of the experiment to follow plant C partitioning and transfer to below ground.

IV.2.4. Plant harvest and soil sampling

Four days after labelling plants were destructively sampled. Subsamples of shoots and roots were freeze dried to determine biomass and soil samples were dried at (80°C, 48 h) before grinding, for further analyses.

IV.2.5. Total C, N and isotope ($^{13}C/^{12}C$ and $^{14}N/^{15}N$) analyses of soil and plant samples

Samples were analyzed for total C and N, as well as isotope ratios ($^{12}C/^{13}C$ and $^{14}N/^{15}N$), using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (IRMS, Sercon Ltd., Cheshire, UK). Samples were combusted at 1020°C in a reactor packed with chromium oxide and silvered colbatous/cobaltic oxide. Following combustion, oxides are removed in a reduction reactor (reduced copper at 650°C). Nitrogen and CO_2 were separated on a

Carbosieve GC column (65°C, 65 mL $min^{-1)}$ before entering the IRMS. Finally, delta ^{15}N and ^{13}C values were measured.

Data are presented in excess ^{15}N and ^{13}C. As compared to the natural abundance (A_N) in plant tissue and soil samples of control plants (^{15}N shoot: 0.370, root: 0.376; ^{13}C shoots: 1.076, roots: ^{13}C 1.079). The total quantity of N and ^{15}N (mg) of a given organ or soil compartment is calculated as follows:

(1) $N_{tot} = BM_{sample} \times \left(\frac{\%N_{sample}}{100} \right)$ [where *BM* = Biomass of the considered plant or soil compartment]

(2) $^{15}N_{total} = N_{total} \times \left(\frac{atom\%^{15}N}{100} \right)$

For calculating ^{13}C we used delta values that were given directly by the mass spectrometer with belemnite as international standard and was calculated as follows:

The $\delta^{13}C$ values are defined as:

(3) $\delta^{13}C = \left(\frac{R_{Sample}}{R_{Standard}} - 1 \right) * 1000;$

where ratio value (R):

(4) $R_{Sample} = \frac{^{13}C}{^{12}C}$

$R_{Standard}$ = 0.01118 (VPDB)

Atom%^{13}C is defined as:

(5) Atom% ^{13}C = 100x F ;

with F the fraction of the heavy isotope:

(6) $^{13C}F = \frac{(\delta + 1000)}{\left[\delta + 1000 + \left(\frac{1000}{R_{S\,\mathrm{tan}\,dard}} \right) \right]}$, according to Frey (2006).

IV.2.6. Soil soluble mineral N (N_{min}) concentration and microbial biomass

Soluble mineral nitrogen in soil was determined in root free soil-subsamples using 6 g fresh weight soil samples. Samples were extracted with 50 ml 0.5 M K_2SO_4 for 1 h at 130 rev min^{-1} and filtered subsequently. Extracted samples were kept frozen until analysis. K_2SO_4 extracts were measured in a Traax 2000 analyser (Bran and Luebbe) for mineral N (N_{min} = NO_3^-N + NH_4^+-N). Mineral N was calculated as

(7) $N_{min} = \left(\frac{N-NH_4 + N-NO_3}{k_{en}} \right)$ with, k_{en} the efficiency constant 0.54 (Brookes *et al.* 1985).

IV.2.7. Size and activity of the soil microbial community

Total numbers of protozoa were enumerated by the most probable number technique (Darbyshire *et al.* 1974). Here 5 g of soil were dispersed in 20 ml NMAS and shaken for 20 min at 75 rpm. Aliquots of 0.1 ml were added to a microtiter plate and diluted two fold in 50 µl sterile NB-NMAS. Microtiter plates were incubated at ca. 15°C and were counted every second day for 14 days starting with the preparation using an inverted microscope. Densities of amoebae were calculated using an automated analysis software (Hurley and Roscoe 1983).

Abundance of arbuscular mycorrhizal fungi was determined from a subsample of fresh roots using the method described by Herdler *et al* (2008). Briefly, fresh roots were boiled in 20 ml 1 N KOH for 1 min in a microwave, acidified with 10 ml 3.7% HCl and stained with a few drops of ink (Quink permanent blue, Parker, Hamburg) for subsequent AM counting by the gridline intersection method (Giovannetti and Mosse 1980). Soil microbial biomass and specific respiration (qO2) were determined from 5 g fresh weight soil as described by Herdler *et al*. (2008). Basal respiration was calculated from the average O_2 consumption rate of samples during 10-20 h after attachment of samples to an automated respirometer based on electrolytic O_2-microcompensation (Scheu 1992). From the same samples we calculated soil microbial biomass by using the maximum initial respiratory response (MIRR) by substrate induced respiration (SIR, Anderson and Domsch 1978) after amendment of 8000 ppm glucose. Glucose was added in aqueous solution increasing the water holding capacity to 100%. The mean of the 4 lowest measurements during the first 10 h after glucose addition were taken as MIRR. Microbial biomass C (C_{mic}, µg g-1) was calculated as 38 x MIRR (µl O_2 h^{-1}) (Beck et al. 1997). Microbial specific respiration (qO2) was calculated from the data on microbial biomass and basal respiration (Scheu 1992).

IV.2.8. Microbial community structure

To analyse phospholipid fatty acids (PLFAs) lipids were extracted from soil according to (Frostegård *et al.* 1993b). Briefly, 4 g of soil (wet weight) were extracted by adding 18.4 ml Bligh and Dyer solvent (chloroform : methanol : citrate buffer of 1:2:0.8, pH 4), vortexed and mixed for 2 h. Samples were centrifuged at 2500 rpm for 10 min and the solvent transferred to new tubes. Samples were re-extracted with 5 ml Bligh and Dyer solvent and the extraction solvents of both steps were combined. To the extract 6.2 ml chloroform and 6.2 ml acid buffer were added, vortexed for 1 min, centrifuged at 2500 rpm for 10 min and allowed to stand for separation. The chloroform fraction (3 ml) of each sample was transferred to a silica acid column (0.5 g silicic acid, 3 ml; HF BOND ELUT – SI, Varian, Inc. U.S.A.) and lipids were eluted with 5 ml chloroform (NLFAs), 10 ml acetone (glycolipids) and 5 ml methanol (PLFAs). PLFA fractions were reduced by evaporation (40 ^{o}C, vacuum 200 hPa) in a vacuum rotator (RVC 2-25, CHRIST ®, Buddeberg, Mannheim). Each sample was dissolved in 1 ml methanol–toluene solvent (1:1) and 30 µl internal standard (5.77 mg methylnondecanoate in 25 ml isooctane) was added. Lipid methanolysis was conducted in 1 ml 0.2 M methanolic KOH (2.8 g KOH in 250 ml methanol) and incubated for 15 min at 37 ^{o}C in a water bath. The FA methyl esters (FAMEs) were extracted with 2 ml hexane–chloroform solvent (4:1), 0.3 ml 1 M acetic acid and 2 ml deionised water. Samples were vortexed and centrifuged at 2500 rpm for 10 min. The organic phase was transferred to new tubes and FAMEs were re-extracted with 2 ml hexane–chloroform solvent. Extraction solvents of both steps were combined and reduced by evaporation. Samples were dissolved in 100 µl isooctane and stored at -20 ^{o}C until analysis. FAMEs were identified by chromatographic retention time comparison with a standard mixture composed of 37 different FAMEs ranging from C11 to C24 (Sigma-Aldrich, St Louis, USA). Analysis was performed by gas chromatography (CLARUS 500 GC) using a GC-FID Clarkus 500 (PerkinElmer Corporation, Norwalk, USA) equipped with HP-5 capillary column (30 m x 0.32 mm i.d., film thickness 0.25 μm). The temperature program started with 60 ^{o}C (held for 1 min) and increased by 30 ^{o}C/min to 160 ^{o}C followed by 3 ^{o}C/min to 260 ^{o}C. The injection temperature was 250 ^{o}C and helium was used as carrier gas. Total PLFAs were determined as the sum of all PLFA biomarker concentrations (nmolg^{-1}, (Zelles 1999a). A sum of 11 PLFAs (12:0, i15:0, a15:0; 16:1ω7, 16:0, cy17:0, 18:2 ω6, 18:1 ω9, 18:0, cy19:0 and 20:4ω6 was used to represent microbial community in soil (Frostegård and Bååth 1996, Zelles 1999b).

IV.2.9. Statistical analyses

Each treatment was replicated 9 times, but CTL, MYC and AMO, MYC treatment 7, 8 and 7 pots were available at the end of the experiment, respectively. Data were analysed by a two-factorial General Linear Model (GLM) procedure with mycorrhiza (without/with) and amoebae (without/with) as factors. For multivariate analysis of variance (MANOVA) the data set was divided into the coherent categories biomass and morphology, soil microbial parameters, and on N, C and ^{13}C and ^{15}N concentrations in *P. lanceolata*. Significant effects of the MANOVA were further explored by separate factorial ANOVAs ("protected" ANOVAs). Normal distribution and homogeneity of variance were improved by log-transformation (log[x + 1]) (Sokal and Rohlf 1995). Statistical analyses were conducted using SAS 9.1 (Statistical Analysis System, SAS Institute Inc., U.S.A.), except the comparison of the overall structure of PLFA profiles between the treatments, that was performed by a discriminant analysis in STATISTICA 7 (Statsoft, Tulsa, U.S.A.).

IV.3. Results

Glomus intraradices successfully colonized the roots of *P. lanceolata* but colonization rates were generally low. Due to partial destruction of roots by boiling in KOH, root colonization by AM fungi could not be quantified. At the end of the experiment, protozoan densities were 870 ± 457 and 2538 ±1600 in the AMO and AMO+MYC treatment, respectively.

Table 11. GLM table of F-values for the effect of amobae and Mycorrhiza on **(A)** *Plantago lanceolata* biomass and morphology, **(B)** soil microbial parameters and **(C)** Carbon (C), Nitrogen (N) and isotope values (^{13}C and ^{15}N) in *P. lanceolata*. Significant F- values of protected ANOVAs in bold (*p<0.05, **p<0.01, ***p<0.001).

A. Plant biomass, leaf area, shoot-to-root ratio of the biomass

		AMO	MYC	AMOxMYC
	d.f.	F	F	F
Plant dry weight	1, 27	**15.68*** **	**7.83** **	0.54
shoot dry weight	1, 27	4.11	3.24	0.88
root dry weight	1, 27	**13.87*** **	**5.55***	3.09
shoot-to-root ratio	1, 27	**7.6***	0.02	**4.41***

B. Microbial biomass, size, activity and structure, soluble N_{min} in soil and belowground (BG) respiration

		AMO	MYC	AMOxMYC
	d.f.	F	F	F
C_{mic}	1, 13	**12.14** **	0.01	0.26
basal resp	1, 13	3.2	0.04	0
qO2	1, 13	3.86	0.08	0.19
tot PLFA	1, 18	**5.39***	**6.0***	1.96
total BG respiration	1, 14	2.73	**7.63***	0.15
$\delta^{13}C$ in soil respiration	1, 13	1.84	**8.19***	2.94
soluble N_{min} soil	1, 26	**4.87***	0.09	0.61

C. Total N, C, plant concentration and isotopic enrichment of ^{13}C and ^{15}N in *P. lanceolata*

		AMO	MYC	AMOxMYC
	d.f.	F	F	F
Tot N	1,27	**6.91***	**5.75***	0.02
Tot C	1,27	**13.39*** **	**7.31***	0.11
C-to-N plant	1, 27	**15.94*** **	**4.6***	0.33
tot ^{15}N plant	1, 25	**17.62*** **	**6.65***	0.01
tot ^{13}C plant	1, 27	**7.63** **	**8.61** **	0.02
atom% ^{15}N plant	1, 30	**12.33** **	0.4	1.85
atom% ^{13}C plant	1, 25	**9.41** **	1.1	1.47

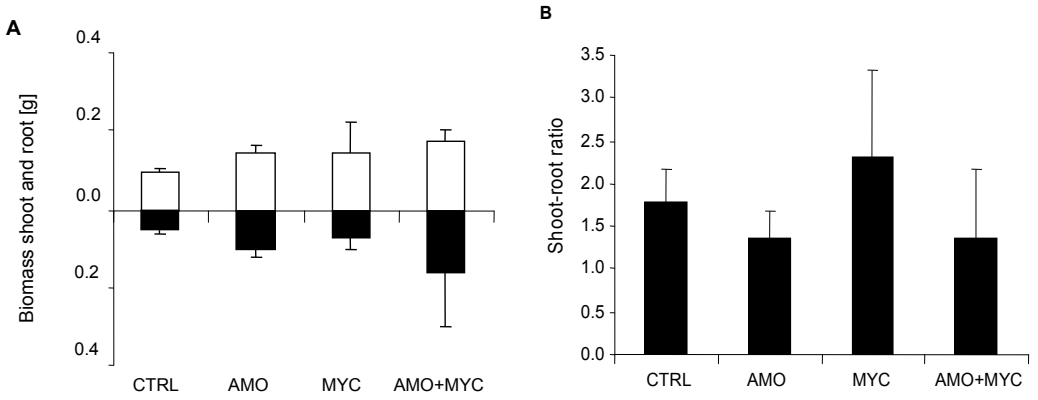

Figure 17. (A) Shoot (white bars) and root (black bars) dry weight (means + 1 SD) and **(B)** shoot-to-root ratio of *Plantago lanceolata* at the end of the experiment in the control treatment (CTRL), either with Amoebae (AMO) or arbuscular mycorrhiza fungi (MYC) alone or both AMO+MYC together.

IV.3.1. Plant biomass and shoot-to-root ratio

Root and shoot biomass increased by a factor of 1.7 and 1.5 in presence of amoebae and similarly by a factor of 1.5 in presence of AM fungi, respectively (Figure 17a). When both amoebae and AM fungi were present, plant biomass increased by a factor of 2.4 (Figure 17a). Irrespective of the presence of AM fungi, the shoot-to-root ratio decreased in the presence of amoebae, indicating a disproportional increase of root growth (Table 11, Figure 17b).

IV.3.2. Total N, atom% ^{15}N and total ^{15}N in *Plantago lanceolata*

Total N in *P. lanceolata* increased in the presence of amoebae and AM fungi and peaked in presence of both (Figure 18a, Table 11). Atom% ^{15}N increased in amoebae and amoeba x AM fungi treatments by a factor of 1.05 and 1.08, respectively (Table 11, Figure 18b).

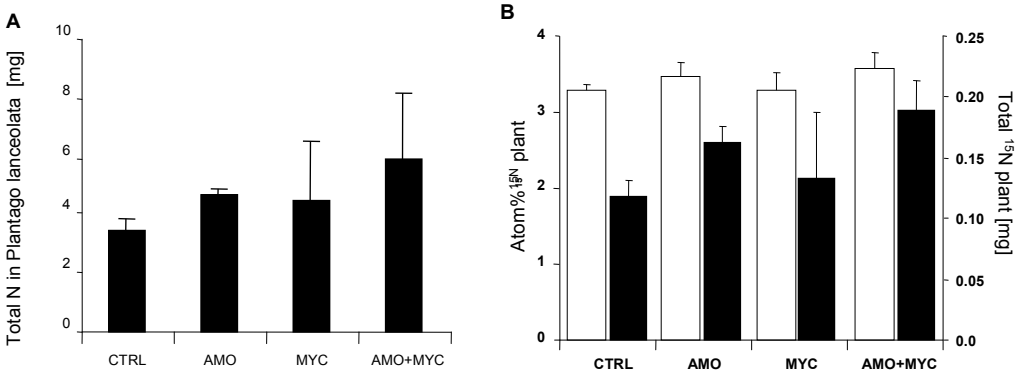

Figure 18. (A) total N and **(B)** atom% ^{15}N (white bars) and total ^{15}N (black bars) in *Plantago lanceolata* at the end of the experiment in the control treatment (CTRL), and treatments with amoebae (AMO) and arbuscular mycorrhizal fungi (MYC; *Glomus intraradices;* means + 1 SD).

Total ^{15}N uptake increased by a factor of 1.38 and 1.12 in treatments with amoebae and AM fungi, respectively (Table 11, Figure 18b). In the presence of both total ^{15}N

uptake was enhanced by a factor of 1.40 with the interaction being not significant. The proportion of total ^{15}N from litter taken up by *P. lanceolata* ranged between 7.4 in the control and 10.22 % in the combined treatment with amoebae and AM fungi, thereby exceeding ^{15}N uptake as reported in experiment 1 of this thesis.

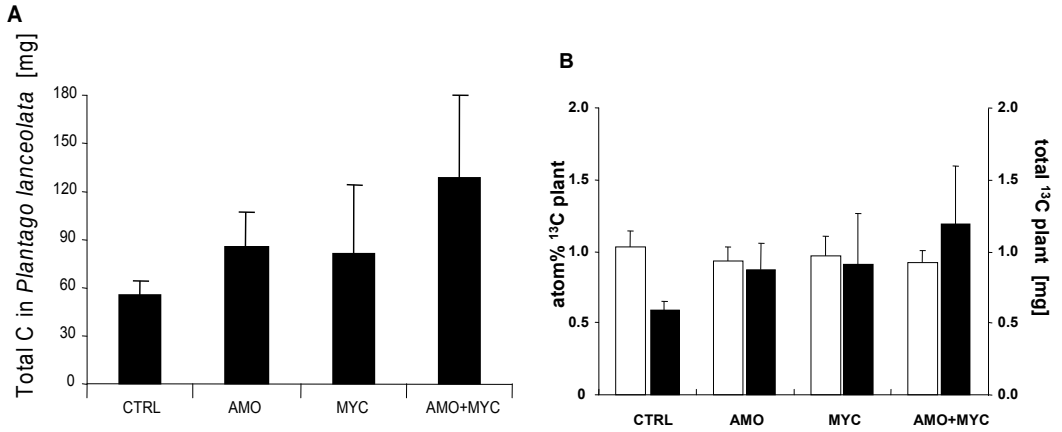

Figure 19. (A) total C, and **(B)** atom% ^{13}C (white bars) and total ^{13}C (black bars) in *Plantago lanceolata* at the end of the experiment in the control treatment (CTRL), and treatments with amoebae (AMO) and arbuscular mycorrhizal fungi (MYC; *Glomus intraradices*; Means + 1 SD).

Total C increased in presence of amoebae and AM fungi and was at a maximum in presence of both (Table 11, Figure 19a). Atom% ^{13}C in *P. lanceolata* decreased in presence of amoebae, whereas AM fungi did not affect atom%^{13}C in the plant (Table 11, Figure 19b).

Total ^{13}C in plants at harvest was enhanced by a factor of 1.5 in presence of amoebae and also by a factor of 1.5 in presence of AM fungi; but increased in presence of both by a factor of 2.2 (Table 11, Figure 19b). Recently fixed C transferred into roots increased by a factor of 1.7 in presence of amoebae and tripled in the presence of both amoebae and AM fungi, representing 35 and 42% of total ^{13}C recovered at harvest, respectively. In the control and AM fungi treatment, ^{13}C in roots represented only 30% and 23% of the total recently fixed C recovered at harvest. Amoebae and AM fungi did not affect total plant atom% ^{13}C and amount of total ^{13}C in an interactive way (Table 11).

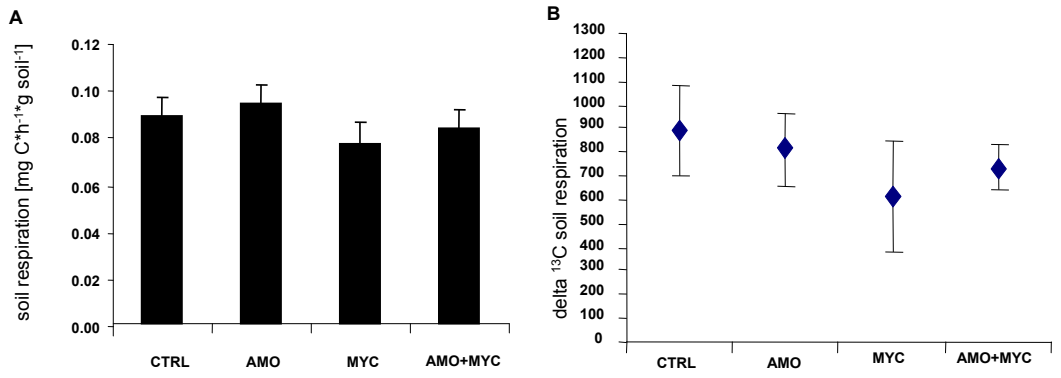

Figure 20. Effects of protozoa and arbuscular mycorhiza on **(A)** total belowground respiration and **(B)** $\delta^{13}C$ in belowground respiration in the control treatment (CTRL), and treatments with amoebae (AMO) and arbuscular mycorrhizal fungi (MYC; *Glomus intraradices*; Means + 1 SD).

IV.3.3. Belowground respiration

The presence of AM fungi reduced total soil respiration to 0.86 of the control; in the combined treatment with AM fungi and amoebae the reduction was somewhat less pronounced (0.95) but the interaction was not significant (Table 11, Figure 20a). Total soil respiration was not affected by the presence of amoebae (Table 11, Figure 20A). The delta ^{13}C value decreased in the presence of AM fungi to 0.68 of the control; again in the combined treatment with AM fungi and amoebae the reduction was somewhat less pronounced (0.82) but the interaction was not significant (Table 11, Figure 20b). The delta ^{13}C value remained unaffected by amoebae (Table 11, Figure 20b).

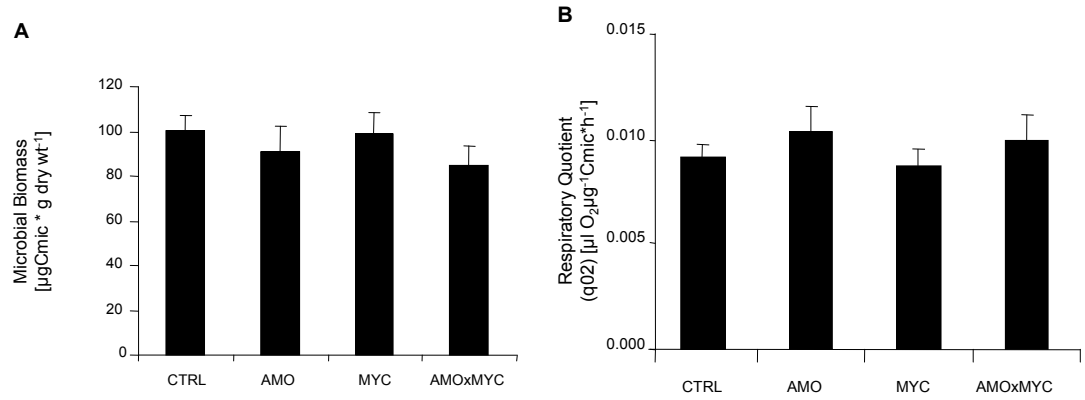

Figure 21. Effects of protozoa and arbuscular mycorrhizal fungi on **(A)** soil microbial biomass and **(B)** specific microbial respiration in *Plantago lanceolata* rhizosphere in the control treatment (CTRL), and treatments with amoebae (AMO) and arbuscular mycorrhizal fungi (MYC; *Glomus intraradices*; Means + 1 SD)

IV.3.4. Soluble N_{min} soil

Concentrations of K_2SO_4 soluble N_{min} (NH_4-N+ NO_3-N) increased in amoebae and amoebae x AM fungi treatments by a factor of 1.49 and 1.45 from 1.39±0.80 µg dry weight soil^{-1} in the control to 2.07±0.72 and to 2.02±0.77 µg^{-1}dw soil^{-1} respectively (Table 11). AM fungi generally did not increase N_{min} concentrations (overall average 1.6±0.25 µg^{-1}dw soil^{-1}, Table 11).

IV.3.5. Microbial biomass, activity and community structure

Amoebae and AM fungi did not affect basal respiration (data not shown,Table 11). In contrast, soil microbial biomass (C_{mic}) decreased 0.85 and 0.84 fold in treatments with amoebae and amoebae x AM fungi, respectively. In parallel specific respiration (qO2) tended to increase by a factor of 1.14 and 1.1 in treatments with amoebae and amoebae x AM fungi (Figure 21a, b).

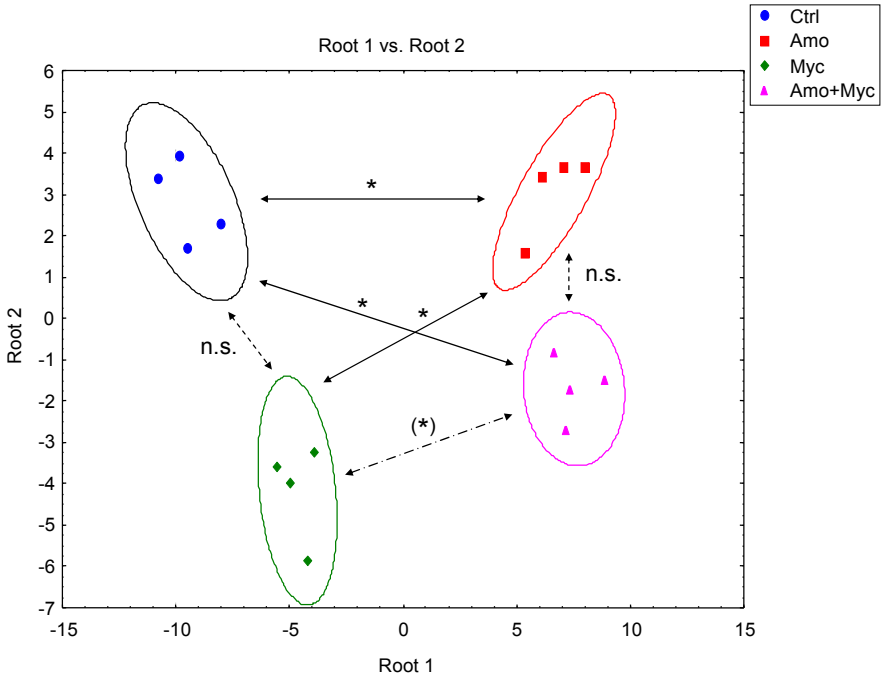

Figure 22. Discriminant function analysis of phospholipid fatty acid (PLFAs) profiles of the soil microbial community in the rhizosphere of *Plantago lanceolata* at the end of the experiment in the control treatment (Ctrl), and the treatments with amoebae (Amo) and arbuscular mycorrhizal fungi (Myc). Ellipses represent 60% confidence limits * $p < 0.05$, (*) = $p < 0.1$.

The amount of the 11 detected PLFAs in soil ranged between 4.8 ± 1.7 in the control, to 6.7±0.9, 6.8±1.2 and 7.41 ± 0.81 nmol g^{-1} dry weight soil amoebae, AM fungi and amoebae x AM fungi treatments, respectively. Amoebae changed microbial community composition compared to control treatments and AM fungi treatments. However, also amoebae and AM fungi treatment differed from each other, with microbial community structure of amoebae x AM fungi treatments being intermediate between AM fungi and amoebae treatments (Figure 22).

IV.4. Discussion

The microcosm system established in this study allowed evaluating plant growth, resource acquisition and partitioning as affected by protozoa – AM fungi interactions in a model plant. Furthermore, the analysis of PLFA patterns allowed exploring shifts in the microbial community structure in response to protozoa–AM fungi–plant interactions. The study therefore for the first time investigated the role of interactions between symbionts of very different phylogenetic association and of contrasting trophic groups (predators and trophic mutualists) in plant N uptake and C partitioning.

In agreement with our hypothesis, protozoa and AM fungi complemented each other in fostering N uptake from litter in soil, leading to maximum plant growth. Protozoa stimulated remobilization of N from bacterial biomass as indicated by increased mineral N in soil and subsequent enhanced amount and concentration of ^{15}N in plants. The resulting increase in plant growth therefore can be attributed to the "microbial loop in soil" (Clarholm 1985, Bonkowski 2004). Interestingly, effect of amoebae on microbial biomass differed as assessed by the SIR and PLFA method: amoebae decreased microbial biomass measured by SIR, but did not affect total amounts of PLFA in microbial biomass. While PLFAs include both, active and inactive microbes, the SIR response is based on metabolic active microorganisms (Dilly 2001, Merila *et al.* 2002, Habekost *et al.* 2008). The decrease in microbial biomass in presence of amoebae as measured by SIR therefore suggests that amoebae preferentially grazed on metabolically active and fast growing rhizobacteria. Rhizobacteria are generally assumed not to be limited by C (Bonkowski 2004, Lu *et al.* 2004) but to rely primarily on N resources for growth (Lynch and Whipps 1990, Nguyen 2003). Our results suggests that rhizosphere microorganisms enhanced the mineralization of N from organic matter in soil and this N pool in part was subsequently remobilized by amoebae grazing. In agreement with Kreuzer *et al.* (2006), Rønn *et al.* (2002b) and Rosenberg (2008), our results show that amoebae strongly shifted microbial community composition, leading to a continuous remobilisation of the microbial N-pool. However, in contrast to amoebae, AM fungi alone did not affect microbial community structure in this experiment. This was surprising, since colonization of roots with mycorrhiza has been shown to be associated to characteristic changes in microbial community structure (Barea *et al.* 2002, Frey-Klett and Tarkka 2007). Probably, the duration of the experiment was too short and the colonisation of the roots by AM fungi too low (as indicated by microscopic inspection) to allow establishment of a microbial community typical for AM fungi. Considering the C-to-N ratio of the litter (15.5), litter-N was likely sequestered by bacteria rather than saprophytic fungi (Hodge et al. 2000). However, an

enhanced plant N uptake from litter in presence of AM fungi suggests that AM fungi effectively competed with bacteria for litter N. Thus, total plant N uptake was fostered *via* an increased absorptive root area by AM fungal hyphae as compared to the control (Marschner and Dell 1994, Smith and Read 1997). Compared to protozoa, the supply of mineral N to the plant by AM fungi was low, suggesting that bacteria were better competitors for litter N than fungi. Short duration of the experiment and low colonization of roots by AM fungi may also explain why AM fungi did not affect ^{15}N concentration in the plants. Since plant N limitation was lowest and plant growth peaked in presence of both root symbionts, amoebae and AM fungi may have complemented each other in plant N foraging.

As argued in our second hypothesis, protozoa and AM fungi indeed altered the partitioning of recently fixed C by the plant. Carbon fixation is known to be modulated by the availability of N (Marschner 1995) and the release of recently fixed C from roots depends on C export from shoots to roots (Swinnen *et al.* 1994a,b, Dilkes *et al.* 2004). In the presence of both amoebae and AM fungi, the amount of recently fixed C in the plants was enhanced and this resulted in increased allocation of this C to roots and into the rhizosphere, thereby stimulating activity and growth of heterotrophic microbes (Cheng and Gershenson 2007). In turn, more N was mobilized for plant uptake. Thus, protozoa and AM fungi complemented each other in plant N acquisition, but also gained the greatest benefit by strongly increasing plant allocation for recent fixed C into rhizosphere. Thus, the interaction of AM fungi and amoebae strongly altered C partitioning in the plant.

Plantago lanceolata adjusts the size of the shoot and root system if the supply of nutrients changes (Grime 1979, Campbell *et al.* 1991, Section III this thesis). In our study, an enhanced N uptake in the presence of AM fungi alone was not accompanied by a reduction in the shoot-to-root ratio as in the treatments with amoebae. Presumably, this was due to a trade off between plant C investment in microbial symbionts and the plants own needs for C (Bonkowski *et al.* 2001, Rønn *et al.* 2002a, Wamberg *et al.* 2003). Especially when nutrients are limiting, plants allocate high amounts of recently fixed C towards AM fungi which can attain up to 30% of recently photosynthates (Smith and Read 1997, Nguyen 2003). Furthermore, *G. intraradices* functions as strong C sink (Lerat *et al.* 2002). Hence, we suggest that AM fungi received high amounts of recently fixed C to build up hyphal networks. As a consequence little of the recently fixed C was exuded by the roots and available for rhizosphere bacteria. This is supported by the fact that AM fungi reduced total soil respiration and the fraction of recently fixed C therein. From a belowground perspective

the results suggest that the stimulated root growth and reduced shoot-to-root ratio of *P. lanceolata* in the presence of both amoebae and AM fungi beneficially affected both rhizosphere symbionts (Bonkowski *et al.* 2001, Kreuzer *et al.* 2006, Herdler *et al.* 2008). Increased C allocation to the roots reduced C limitation of the both symbionts and in turn, they enhanced mobilization of N for plant uptake.

In agreement with our last hypothesis, protozoan grazing shaped microbial community structure. Differences in microbial biomass as measured by PLFA and SIR suggest that protozoa predominantly grazed on metabolically active and fast growing rhizobacteria. Thereby, amoebae enhanced bacterial turnover and shifted microbial community structure. In contrast to protozoa, AM fungi did not affect microbial community structure which presumably was due to low root colonization and biomass of AM fungi. This indicates that in our experiment the potential benefits of AM fungi for plant growth were not fully realized. To explore this potential experiments lasting longer are necessary enabling mycorrhiza to more extensively colonize plant roots and to build up extraradical hyphal networks.

IV.4.1. Conclusions

Overall, our results show high plasticity of plants response to rhizosphere symbionts for maximising N and C foraging. The experiment established *P. lanceolata* as suitable model plant to analyse the role of protozoa-AM fungi interactions in host plant C and N acquisition and allocation. Our results show that uptake from litter is crucial for plant growth and that plant invests high amounts of C for N acquisition. The amount of C allocation is specifically mediated by symbionts. Protozoa and AM fungi complemented each other in nutrient acquisition: protozoa increase N mobilization and AM fungi nutrient capture and transport. Carbon partitioning to symbiotic microorganisms was at a maximum in the presence of both, AM fungi and protozoa, resulting in a mutual interactions for both, the plant and microbial symbionts. Dissecting the mechanism of AM fungi and protozoa interactions is assumed to be the key for understanding processes of plants N acquisition. To further explore protozoa – AM fungi – plant interactions, root and hyphal pathways in N acquisition were separate in the following experiment.

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Chapter V. Protozoa and arbuscular mycorrhiza complement each other in plant nitrogen nutrition from a nutrient patch

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Summary

- In most terrestrial ecosystems nitrogen is the primary limiting nutrient for plant growth. Plants interact with multiple symbionts to improve nitrogen acquisition and growth.
- Free living symbionts, such as protozoa, mobilize N locked up in bacterial biomass, and root infecting symbionts, such as arbuscular mycorrhizal (AM) fungi, transport nutrients extracted from soil to plants in exchange for photosynthetically fixed carbon. The objective of this study was to investigate whether AM fungi and protozoa complement each other in N acquisition from organic residue patches distant to the host plant
- Using ^{13}C and ^{15}N isotope labelling, we show that protozoa and AM complement each other to synergistically promote carbon allocation to and nitrogen allocation from patches of organic matter inaccessible to roots.
- The results document that multiple mutualistic partners, including protozoa and AM are necessary for optimizing plant nitrogen nutrition by exploitation of resources in distance to roots,

V.1. Introduction

Mineral nitrogen limits plant growth in most terrestrial ecosystems (Vitousek and Howarth 1991). Although plants interact with multiple root infecting and free living symbionts to meet their need for mineral nitrogen (van der Heijden *et al.* 2007), only few studies included more than one symbiont when studying plant-mutualist interactions (Bonkowski 2004). As the functioning of symbionts may differ fundamentally when imbedded in multiple interactions (Stanton 2003, Wamberg *et al.* 2003, Strauss and Irwin 2004) plant-mutualist interactions need to be studied under more natural settings. Arbuscular mycorrhizal (AM) fungi, the oldest and most

important plant mutualist (Brundrett 2002), contribute to host plant phosphorous and nitrogen (N) acquisition from soil and organic matter (Hodge *et al.* 2001) in exchange of plant carbon (Jones *et al.* 2004). Thereby AM fungi successfully compete with other rhizosphere microorganisms for mineral N (Tibbet 2000) and subsequently translocate it to the root for plant uptake (Govindarajulu *et al.* 2005). Thus, AM fungi primarily functions are to extend the space from which nutrients are extracted and to accelerate the transport to roots. However, other root colonizing and free living rhizosphere symbionts also significantly contribute to plant N nutrition; e.g., by feeding on bacteria, protozoa mobilize N locked up in bacterial biomass ('microbial loop' in soil) (Clarholm 1985) thereby rendering it available for transport to plant roots via AM hyphae (Bonkowski 2004). However, little is known on how AM fungi and soil protozoa interact in host plant N nutrition. In this study we tested the hypothesis whether AM fungi and protozoa complement each other in N acquisition from organic residue patches distant to the host plant. To establish a diverse microbial community resembling that in the field, we re-inoculated sterilized soil with a bacterial filtrate from rhizosphere soil. Plant symbionts were inoculated by adding axenic protozoa (*Acanthamoeba castellanii*) and axenic AM fungi (*Glomus intraradices*) to the rhizosphere of the host plant *Plantago lanceolata*. The experiment was set up in two-compartment microcosms with plant roots being confined to one compartment (root compartment) while the second compartment contained a patch of ^{15}N labelled plant residues (patch compartment) (Figure 23).

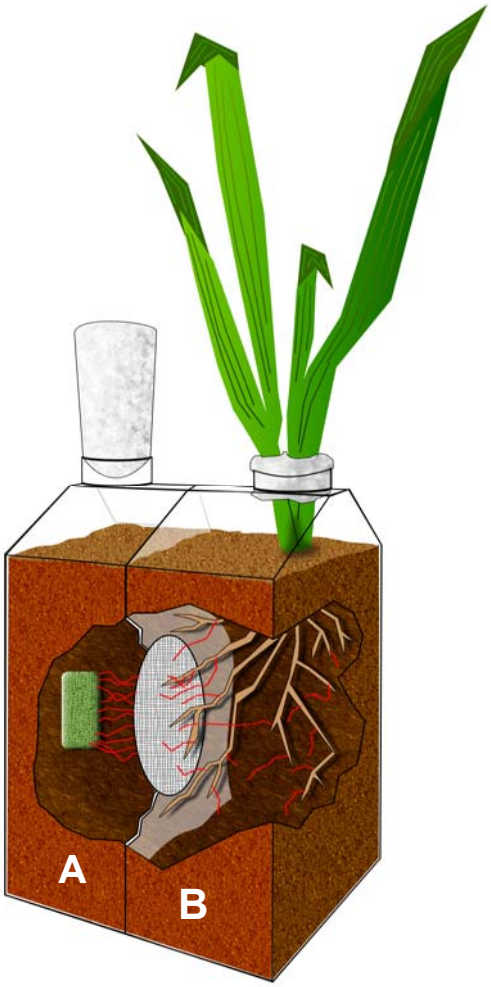

Figure 23. Microcosm set-up. Microcosms were separated into two compartments: a patch compartment (A) and a root compartment (B). *Plantago lanceolata* growing in the root compartment was colonized by arbuscular mycorrhiza *Glomus intraradices*. Both compartments were either separated by a double layer mesh with 0.45 (no access of hyphae to the patch compartment) or 20 µm pore size (access of hyphae to the patch compartment). Plant roots were generally confined to the root compartment. The red lines in the compartments indicate arbuscular mycorrhizal hyphal mycelium; brown lines indicate roots and green rectangle nutrient patch

The compartments were either separated by a double layer of 0.45 µm membrane which neither roots nor mycorrhizal hyphae could penetrate (control), or by a 20 µm mesh, allowing AM fungi but not roots access to the patch compartment via extraradical hyphae. By excluding roots from the patch compartment, N transfer to plants was limited to extraradical hyphae of AM. To analyse if protozoa increase the capture of N by AM fungi and therefore plant N acquisition from the patch, the patch compartment was either set up with mycorrhiza only or with both AM and *A. castellanii* . The latter treatment investigated whether N mobilized by the microbial loop was captured by AM fungi and transferred to the plant ('patch effect of protozoa'). Further, by adding protozoa to both, the root and patch compartment, we analysed if the presence of protozoa in the vicinity of roots modulates plant – mycorrhiza interactions

and therefore plant N acquisition from the patch ('rhizosphere effect of protozoa' (Bonkowski 2004). AM fungi receive up to 20 % of the net fixed carbon (C) from plants (Smith and Read 1997) allowing them to exploit nutrients in soil (Heinemeyer *et al.* 2006); by pulse-labelling the plants with $^{13}CO_2$ we intend to prove that AM fungi *via* extraradical hyphae translocate plant carbon into the patch for nutrient mobilisation.

V.2. Material and Methods

V.2.1. Microcosms and labelling procedure

The microcosms consisted of two 250 ml cell culture flasks, one containing the mycorrhizal host plant, the other the organic residue patch. Openings (5.5 cm diameter) on one side of the flasks were facing each other, connecting both compartments. Each opening was sealed by a mesh (see above) to prevent access to the patch compartment by plant roots (all treatments) or AM fungi hyphae (control treatment). Each sterilised compartment was filled with 320 g autoclaved (20 min, 121 °C) soil-sand mixture (50:50). A natural protozoa-free bacterial inoculum[5] was prepared by subsequent filtering the supernatant of a soil slurry though 5 and 1.2 µm filters (Bonkowski and Brandt 2002). Each of the compartments received 8 ml of the suspension. After washing in sterile water, protozoa treatments were inoculated with 200 µl (approximately 400,000 individuals) of *A. castellanii.* Non-protozoa treatments received 200µl of mineral water instead. Milled ^{15}N labelled *L. perenne* litter (45.2 atom% ^{15}N, 40.8% C, 2.7 % N) was mixed with non-labelled *L. perenne* litter (39.8% C, 2.4% N) to obtain litter containing 10 atom% ^{15}N. From this material 1.0 g was mixed with 9.0 g of soil and filled into a mesh bag (pore size 20 µm), used as organic residue patch. After autoclaving one bag was placed in the centre of the non planted compartment. *P. lanceolata* seeds were surfaced sterilized (Hensel *et al.* 1990) and exposed for germination into Petri dishes. Five days after germination they were transferred into sterile tubes filled with quartz sand and inoculated with a 4 mm³ large piece of agar containing spores and mycelium of the axenic AM. These tubes were transferred to the top of the plant compartment. The young plants were protected from contaminations by air borne cysts of protozoa by a transparent centrifuge tube. Plants were grown in a climate chamber at 18 / 22°C night/ day temperature, 70% of humidity, 16 h of photoperiod, 460 ± 80 µmol m^{-2} s^{-1} light photon flux density in the PAR range. Soil moisture was maintained gravimetrically at 75% of field capacity. Five weeks old

[5] Bacterial inoculum may have contained other soil organism than bacteria, e.g. spores of soil fungi

plants were transferred to an assimilation chamber for 24 h for subsequent labelling. Climatic conditions were the same as above. Plants were pulse labelled with 50 atom% $^{13}CO_2$ for 5 h (Robin 2007). Three unlabelled plants per treatment were maintained in the growth chamber for determination of natural ^{13}C abundance in plant organs and soil compartments. Prior to destructively sampling four days after labelling, net CO_2 assimilation rate was measured on the apical part of a mature single leave with an infrared gas analyser and photosynthetic leaf cuvette (PP system CIRAS-1).

V.2.2. Analytical procedures

Shoot and root samples were freeze dried to determine biomass. Soil samples from patch and root compartments were dried (80 °C, 48 h). Plant tissue and soil samples were milled to fine powder for analysis by an elemental analyser (Carlo Erba, Na 1500 type II, Milan, Italy) coupled with an isotope mass spectrometer (Finnigan Delta S, Bremen, Germany). Data were presented in ^{15}N and ^{13}C in excess of the natural abundance. Soluble C in soil from the patch compartment were determined using the method described by Henry *et al.* (2005). AM abundance was determined from a subsample of roots before drying (Phillips and Hayman 1970). For 16:1ω5 phospholipid fatty acid (PLFA) profile extracting we used a using modified Blight and Dyer-method (Bligh and Dyer 1959) and gas chromatography analysis (Gormsen *et al.* 2004) (for more details see Chapter III & IV this thesis).

V.2.3. Statistical Analysis

Differences between the treatments were analysed by a General Linear Models (GLM) procedure in SAS (v. 9.1) using four contrasts:

(1) Control *versus* mycorrhiza only treatment (no hyphal access to the organic residue patch *versus* hyphal access to the organic residue patch; data not shown) analysing the effect of mycorrhiza

(2) Mycorrhiza only *versus* mycorrhiza + amoebae in patch compartment (hyphal access to the organic residue patch in absence *versus* presence of amoebae in the organic residue patch) analysing the patch effect of protozoa

(3) Mycorrhiza only *versus* mycorrhiza + amoebae in patch and root compartment (hyphal access to the organic residue patch in absence *versus* presence of amoebae in both, the organic residue patch and the rhizosphere) analysing the overall effect of protozoa

(4) Mycorrhiza and amoebae in patch compartment *versus* mycorrhiza + amoebae in patch and root compartment (hyphal access to the organic residue patch in presence of Amoeba in the organic residue patch *versus* presence of amoebae in both, the organic residue patch and the rhizosphere) analysing the root effect of protozoa.

V.2.4. Results and Discussion

Access of AM fungi to the patch compartment neither affected shoot ($F_{1,22}$ = 2.27 p = 0.15) nor root biomass ($F_{1,19}$ = 0.75, p = 0.40, data not shown). However, access of AM fungi to the patch significantly increased total soil ^{13}C signature in the patch substrate from -29.6±0.4 to -28.7±0.5 ($F_{1,22}$ = 12.18, p = 0.0025) confirming that plant C was translocated *via* extraradical hyphae into the patch. Despite AM fungi colonized the patch they did not significantly increase ^{15}N uptake by the host plant shoot and roots ($F_{1,22}$ < 0.01, p = 0.95 and $F_{1,19}$ = 0.27, p= 0.61, respectively; data not shown), demonstrating that AM fungi were unable to decompose organic ^{15}N in the patch, during the short running time of the experiment. In contrast, if AM fungi and protozoa were present in the patch compartment the amount of ^{15}N in plant shoots increased by a factor of 1.6 ($F_{1,22}$ = 4.40, p = 0.049) (Figure 24a) but the amount of ^{15}N in roots remained unaffected ($F_{1,22}$ = 0.54, p = 0.4712) (Figure 24a). Obviously, protozoa increased the capture of N from organic residues by AM fungi suggesting that the two symbionts complemented each other in plant N nutrition.

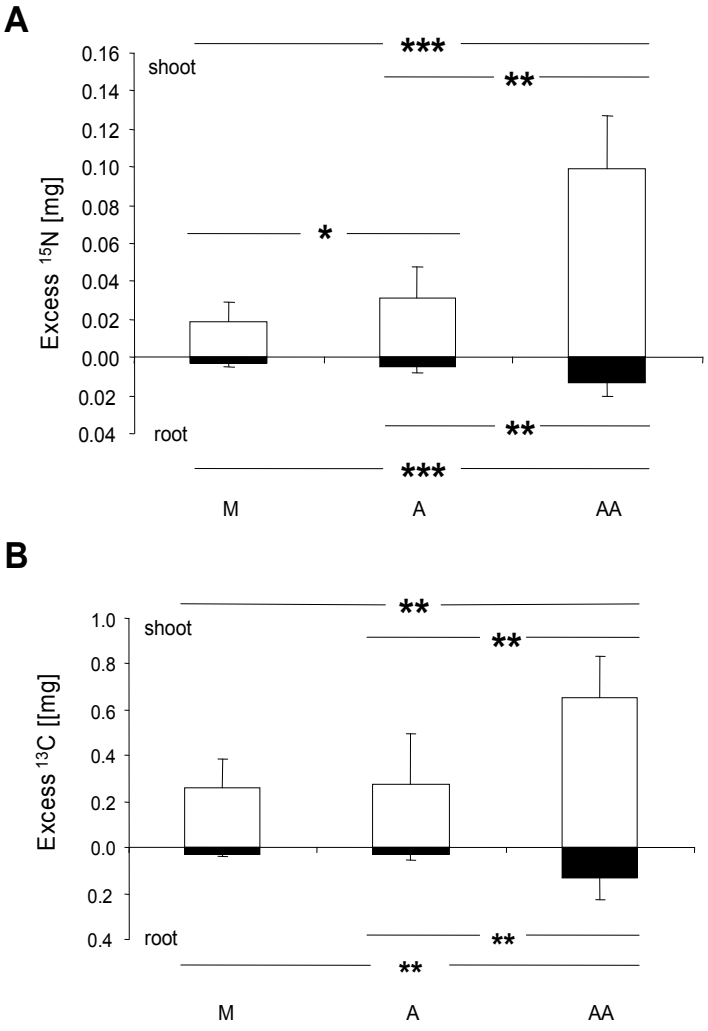

Figure 24 Excess total ^{15}N **(A)** and ^{13}C **(B)** in Plantago lanceolata shoots (open bars) and roots (black bars). Differences between treatments were analysed by General Linear Models (GLM): Mycorrhiza only (M) versus mycorrhiza and amoebae in patch compartment (A) (hyphal access to the organic residue patch in absence versus presence of Amoeba in ORP), mycorrhiza only versus mycorrhiza + amoebae in patch and root compartment (AA) (hyphal access to the organic residue patch in absence versus presence of amoeba in both, organic residue patch and rhizosphere), mycorrhiza + amoebae in patch compartment versus mycorrhiza + amoebae in patch and root compartment (hyphal access to the organic residue patch in presence of amoeba in the organic residue patch versus presence of amoeba in both, ORP and rhizosphere). Error bars are one standard deviation from the mean. Stars indicate significant differences at * = P<0.05; ** = P<0.01; *** = P<0.001. (A) Excess 15N [mg] in shoots and roots: Contrast analysis (GLM) for shoots indicates increase for M vs. A ($F_{1,22}$ = 4.40, p= 0.0495), M vs. AA ($F_{1,22}$ = 28.84, p <.0001) and A vs. AA ($F_{1,22}$ = 8.60, p = 0.0085). Contrast analysis (GLM) for roots confirmed increase for M vs. AA ($F_{1, 19}$ = 16.79, p = 0.0006), and A vs. AA ($F_{1, 19}$ = 9.82, p = 0.005) but not M vs. A (F1, 19 = 0.54, p = 0.471) (M n = 6 ,A n= 6, AA n =7). (B) Excess ^{13}C [mg] in shoots and roots: Contrast analysis (GLM) for shoots indicates increase for shoot M vs. AA ($F_{1,22}$ = 15.03, p = 0.001) and A vs. AA ($F_{1,22}$ = 9.66, p = 0.006), but not for M vs. A. ($F_{1,22}$ = 0.31, p = 0.585). Contrast analysis (GLM) for roots confirmed increase for M vs. AA ($F_{1, 19}$ = 9.74, p = 0.006) and A vs. AA ($F_{1, 19}$ = 8.80, P = 0.008) but not for M vs. A ($F_{1, 19}$ = 0.02, p = 0.888) (M, n = 6; A, n = 6; AA, n =7).

Presumably, conform to the microbial loop, protozoa mobilized N from consumed bacterial biomass thereby making it available for uptake by AM hyphae which subsequently translocated patch derived N to the host plant (‘patch effect of protozoa’). However, increased plant N uptake did not result in increased plant biomass (Figure

25). In contrast to the treatment where protozoa were confined to the patch compartment, shoot and root biomass increased by a factor of 1.8 ($F_{1,22}$ = 7.66, p = 0.0123) and 3.7 ($F_{1,19}$ = 10.66, p = 0.0041) when protozoa were also present in the root compartment (Figure 25). In this treatment uptake of ^{15}N in plant biomass increased two- and five fold compared to treatments with protozoa and mycorrhiza or only mycorrhiza in the patch compartment contributing 15 % of total N to the host plant. The strong increase of plant growth by protozoa suggests that the 'rhizosphere effect' of protozoa exceeded their 'patch effect'.

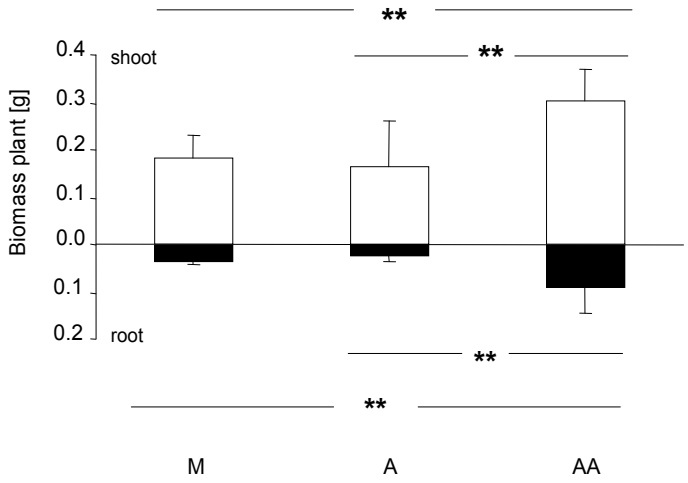

Figure 25 Dry weight [g] of *Plantago lanceolata* shoots (open bars) and roots (black bars) colonised by *Glomus intraradices*. The presences of Protozoa in the patch and rhizosphere compartment increased shoot and root biomass. Differences between the treatments were analysed by General Linear Models(GLM): Mycorrhiza only (M) *versus* mycorrhiza and amoebae in patch compartment (A) (hyphal access to the organic residue patch in absence *versus* presence of Amoeba in ORP), mycorrhiza only *versus* mycorrhiza + amoebae in patch and root compartment (AA) (hyphal access to the organic residue patch in absence *versus* presence of amoeba in both, organic residue patch and rhizosphere), mycorrhiza + amoebae in patch compartment *versus* mycorrhiza + amoebae in patch and root compartment (hyphal access to the organic residue patch in presence of amoeba in the organic residue patch *versus* presence of amoeba in both, ORP and rhizosphere). Error bars are one standard deviation of the mean. Stars indicating significant differences at * P<0.05; ** P<0.01; *** P<0.001. Contrast analysis (GLM) indicates increased plant biomass in shoot AA vs. M and AA treatments: M vs. AA ($F_{1,22}$ = 10.18, p = 0.005) and A vs. AA ($F_{1,22}$ = 7.66, p = 0.012) but not for M vs. A ($F_{1,22}$ = 0.15, p = 0.699) and in roots for M vs. AA ($F_{1,19}$ = 7.86, p = 0.012) and A vs. AA ($F_{1,19}$ = 10.66, p = 0.004) but not for M vs. A ($F_{1,19}$ = 0.18, p = 0.674) (M, n = 6; A, n = 6; AA, n = 7).

Compared to when amoebae were confined to the patch compartment, their presence in the rhizosphere enhanced plant C foraging as measured by net CO_2 assimilation rates by a factor of about two, from 2.78 ± 1.45 to 5.89 ± 0.28 µmol m^{-2} s^{-1} ($F_{1,19}$ = 20.97, p = 0.0003). As a consequence, the pool of recently fixed ^{13}C in *P. lanceolata* tripled (Figure 24b). This most likely explains the increased root colonization by AM

fungi (Figure 26) since AM fungi receive mostly recently fixed C from its host plant (Jakobsen and Rosendahl 1990).

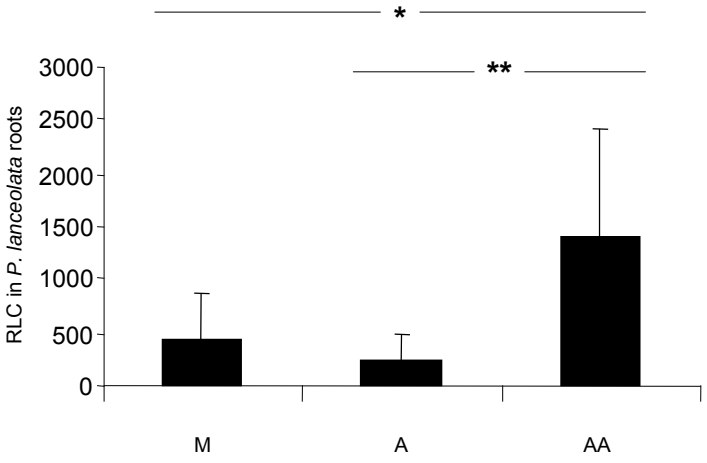

Figure 26. Total root length colonization (RLC) of *Plantago lanceolata* with arbuscular mycorrhiza (*Glomus intraradices*). Presence of Protozoa in the patch and rhizosphere compartment increased RLC. Differences between the treatments were analysed by General Linear Models (GLM): Mycorrhiza only (M) *versus* mycorrhiza and amoebae in patch compartment (A) (hyphal access to the organic residue patch in absence *versus* presence of Amoeba in ORP), mycorrhiza only *versus* mycorrhiza + amoebae in patch and root compartment (AA) (hyphal access to the organic residue patch in absence *versus* presence of amoeba in both, organic residue patch and rhizosphere), mycorrhiza + amoebae in patch compartment *versus* mycorrhiza + amoebae in patch and root compartment (hyphal access to the organic residue patch in presence of amoeba in the organic residue patch *versus* presence of amoeba in both, ORP and rhizosphere).Error bars are one standard deviation of the mean. Stars indicating significant differences at * P<0.05; ** P<0.01; *** P<0.001. Contrast analysis in (GLM) indicates increased RLC for M vs. AA ($F_{1, 23}$ =7.97, p= 0.01) and A vs. AA ($F_{1, 23}$ = 10.66, p = 0.004) but not for M vs. A ($F_{1, 23}$ =0.32, p= 0.579) (M, n = 6; A, n= 6; AA, n =7).

Increased C supply for AM fungi likely increased the extraradical hyphal network thereby contributing to a more pronounced exploitation of nutrients in the patch compartment. Indeed, the maker PLFA for AM fungi (Olsson *et al.* 1995), 16:1ω5, increased more than five fold in the patch from 0.91± 0.65 in the treatment with amoebae confined to the patch compartment to 6.26 ± 7.19 nmol g^{-1} dw soil in the treatment with amoebae also in the root compartment ($F_{1,13}$ = 4.71; p = 0.055). Parallel to the biomass of AM fungi carbon in the patch was enriched in ^{13}C in presence of AM fungi (-27.72±0.22 to -26.95± 1.03 delta units without and with AM fungi, respectively; $F_{1,21}$=5.07, p = 0.037) suggesting that they translocated plant C into the patch. This increased C in the patch triggered microbial activity (De Nobili *et al.* 2001) and increased specific respiration[6] of microorganisms from 0.023 ± 0.007 to 0.033 ± 0.011 µl O_2 μg^{-1} C_{mic} h^{-1} ($F_{1,20}$ = 4.92, p = 0.04). This induced "priming effect" (Kuzyakov 2002)

[6] microbial specific respiration quotient (qO2) is the ratio of microbial respiration per unit biomass

subsequently stimulated decomposition of organic matter in the patch and hyphal uptake of mineral nitrogen for host plant nutrition.

Separating the patch from the rhizosphere effect of protozoa uncovered complementarily of rhizosphere symbionts i.e., proved that protozoa and AM fungi synergistically increase C and N acquisition of the host plant. When confined to the patch of organic matter protozoa increased N mobilization presumably *via* the 'microbial loop' mechanism. When also present in the root compartment protozoa stimulated photosynthesis of the host plant and the translocation of recently fixed C to AM fungi. This fostered nutrient acquisition by AM fungi *via* exploitation of nutrient rich hotspots by an increased network of extraradical hyphae showing that mycorrhizosphere is crucial for plant nutrient acquisition (Barea *et al*. 2002). Increased plant derived C translocated by extraradical hyphae into the patch enhanced microbial food web functioning and thereby organic nutrient mineralization. Mineral nitrogen was subsequently taken up by AM fungi and transferred *via* extraradical hyphae to roots resulting in increased plant nutrition and plant growth. Complementary function of protozoa and AM fungi in delivering plant nutrients from patches in distance to roots suggests that plant growth and interactions in plant communities can only be understood when considering multiple rhizosphere mutualists, i.e., when appreciating the complexity of rhizosphere interactions in heterogeneous soil.

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Chapter VI. General discussion

Grasslands are important terrestrial ecosystems covering about a quarter of the Earth's land surface. Soil systems in grasslands differ from other vegetation types because of high turnover of shoot and root biomass (Wardle 2002). The dynamics of grassland plant communities is shaped by the individual plants' ability to compete successfully for soil resources to improve growth (van der Krift *et al.* 2001). Consequently, the rhizosphere is a strategic point where plants manipulate microbial communities to improve nutrient availability (van der Heijden *et al.* 2007). In turn, rhizosphere microbes have the ability to shape plant performance (Kiers and van der Heijden 2006). Thus, plants and the decomposer subsystem are highly connected and this forms the basis for soil fertility and ecosystem productivity

Until recent, little attention was paid to the role of interacting symbionts on plant assimilate partitioning and nutrient availability. This is surprising since plants invest significant amounts of photoassimilates for enhancing nutrient nutrition and fostering plant growth.

The present thesis investigated how amoebae in the rhizosphere and their interactions with AM fungi affect nitrogen availability for plants. Plant responses were detailed by investigating feed backs on C assimilate partitioning and C allocation to root symbionts. Further, feedbacks on microbial community structure were assessed.

This general discussion will argue the model system and the major results obtained using this system. Based on these results suggestions for improving agriculture practices are given. Finally, pros and cons of the methodologies used in this study are discussed and suggestions for future experiments are provided. Figure 27 summarizes major achievements and outlooks of this general discussion.

The experiments of this thesis were performed in the framework of the concept of the “microbial loop in soil” (Clarholm 1985). Next to nematodes, protozoa, are the most prominent bacterial grazers and, consequently, were chosen as model symbionts. Protozoa are free living, common and abundant in the rhizosphere of plants, representing strong bacterial grazers in the microbial food web (Bonkowski 2004). Additionally, I have chosen AM fungi that colonize roots of more than 80% of all plant species and live as obligate biotrophs. For both symbionts, protozoa and AM fungi, plant growth promoting effects have been reported and attributed to the enhancement of plant nutrient supply (Smith and Read 1997, Bonkowski 2004). Only few studies

investigated their interactions (Wamberg *et al.* 2003, Herdler *et al.* 2008). In order to assess in detail plant nitrogen acquisition and its feedbacks on C photoassimilate partitioning, we focussed on single plant model systems. Stable isotopes were applied to trace C (^{13}C) and N (^{15}N) partitioning in the plant soil system.

In contrast to plant-amoebae interactions, it is known that the interplay between plants and AM fungi determines the plant-AM fungi symbiosis (Johnson *et al.* 1997). Consequently, the first aim of this thesis was to test potential model plants for morphological and nutritional responses induced by amoebal grazing on microbial community **(Chapter 2)**. Significant responses were crucial for the choice of the plant species used for the following studies in this thesis, since plant responses were used as indicators for favourable or unfavourable environmental growth conditions. The tested plant species indeed varied in their morphological responses to the presence of amoebae in the rhizosphere. In the responding plants morphological shifts indicated improved growth conditions (e.g. increased specific leaf area). Consequently, plants may select microbial symbionts for mineral nutrient uptake from organic matter (litter) added to soil. Based on these results follow up experiments were performed to dissect the mechanisms of plant-amoebae interactions for N nutrition.

The role of litter quality (defined by plant tissue C-to-N ratio) for plant N nutrition *via* protozoan activity was examined **(Chapter 3)**. To investigate this dependency I have chosen litter with contrasting C-to-N ratios and investigated if N is either sequestered into or liberated from microbial tissue. Amoebae mobilized N from bacterial biomass independent of the litter C-to-N ratio. This underlines that next to saprotrophic fungi protozoa contribute significantly to rhizosphere nutrient fluxes *via* grazing on bacterial biomass. Remarkably, this was also the case in presence of more recalcitrant litter substrates which underlines the generality of the microbial loop concept, i.e. that plant derived C resources fuel microbial interactions to stimulate N mobilization (Figure 27). Plant C allocation and rhizodeposition was modified by both, litter quality and the presence of amoebae. In treatments with recalcitrant litter material plants allocated a higher proportion of recently fixed photosynthates into the rhizosphere, as compared to treatments where N was easier to access. However, only in the presence of amoebae the enhanced C allocation to the rhizosphere was beneficial for the plants, as indicated by enhanced plant growth. Further, with recalcitrant litter, grazing by protozoa altered the structure of microbial communities which may have contributed to increased plant growth by favouring plant growth promoting bacteria. If N in litter was easily accessible, plant and microbial biomass exceeded that of recalcitrant litter treatments and plants assimilated more C and N for biomass production. Also, here, amoebae beneficially

affected plant growth. Microbial community was not affected suggesting that where net N mobilization occurred beneficial traits of amoebae were predominantly due to an additional N mobilization.

In the field, plants are associated with multiple root infecting and free living symbionts of different phylogenetic affiliations and trophic levels. In contrast to this situation, most studies investigating plant-symbiont interactions are restricted to pairwise plant-mutualist interactions. The role of symbionts as revealed by single symbiont-plant observations may be misleading, since interactions of species with functional dissimilar traits may foster or cancel out each other (Wurst *et al.* 2008). In our model system we examined whether protozoa and AM fungi interact in fostering plant nutrition and growth **(Chapters 4 & 5).** Indeed, AM fungi and amoebae complemented each other in plant N uptake. This was true for both homogeneously and patchy distributed litter into soil (Figure 27). Furthermore, protozoa-AM fungi interactions synergistically increased plant growth when exploiting N from litter patches distant to roots. By tracing C and N fluxes, the study showed that AM fungi translocate plant derived C into the patches, thereby stimulating microbial activity. In the root free litter patch AM fungi successfully competed with other microbes for N mobilized by protozoa and allocated it to the plant host. Thus, AM hyphae acted as pipe system allocating C and N from source to sink regions, thereby affecting the functioning of saprotrophic microorganisms (Figure 27). Additionally, amoebae in the rhizosphere of plants further stimulated plant growth by fostering the exploitation of nutrients in the patch, thereby enhancing the functioning of AM fungi, i.e. increasing the transfer of N *via* mycorrhizal hyphae from the patch to the plant. Thus, our results suggest that the presence of protozoa allowed the plant to link the bacteria and mycorrhiza based channel of decomposer systems, thereby optimizing growth. This indicates that traits of symbionts beneficial for plant growth were only exploited to the full benefit of the plant when imbedded in multitrophic interactions.

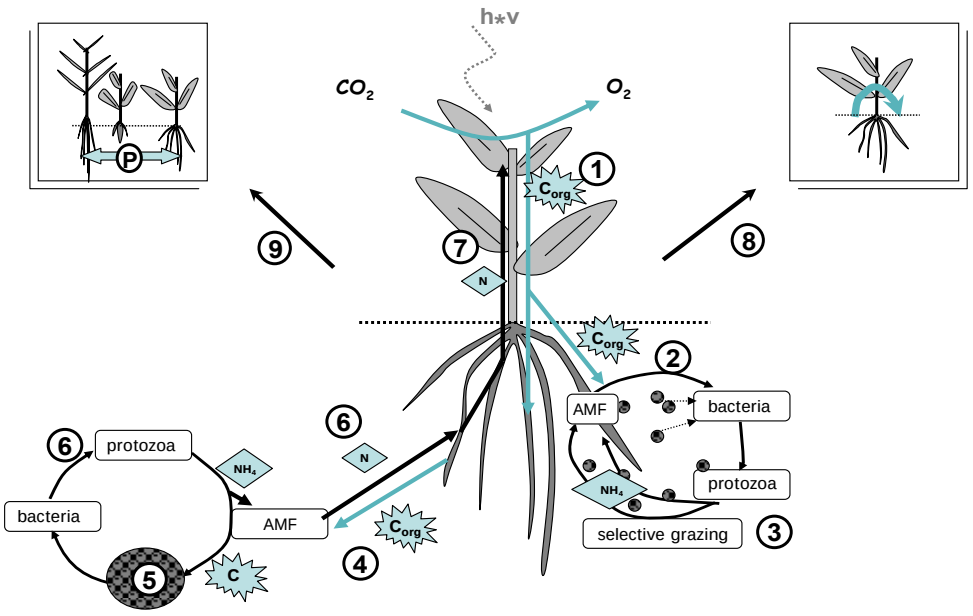

Figure 27. Multitrophic interactions in the rhizosphere: (1) Plants fix C for belowground allocation. (2) C rhizodeposition stimulates bacterial growth and activity in the rhizosphere, thereby sequestering N from homogeneously distributed litter. Protozoa remobilize N from bacterial biomass independent of litter quality and (3) change bacterial community composition by selective grazing. Both, plant growth and morphological responses vary with plant species. Arbuscular mycorrhizal fungi (AMF) enhance nutrient uptake by increasing root absorptive surface. (4) Plants allocate C into extraradical hyphae of AMF, (5) draining C into soil and nutrient patches. Thereby, AMF trigger microbial activity and consequently N mineralization in patches distant to roots. (6) In turn to C supply, N mobilized by protozoa is transferred *via* extraradical hyphae to the host plant and enhances plant N nutrition and thereby plant growth. Both AMF and protozoa complement each other in fostering plant N nutrition from homogeneously and patchy distributed litter. (8) Whether AMF and protozoa interactions on C allocation are systemically or locally mediated, needs further studies. (9) Moreover, the role of protozoa–AMF interactions for plant nutrient acquisition and C allocation needs to be studied in plant communities.

It is likely that the increase in nutrient mobilization and plant growth in presence of AM fungi (Hodge *et al.* 2001, Barea *et al.* 2002) has to be at least partly attributed to protozoa. Indeed, mycorrhizal inocula used in those studies may have contained protozoa. AM fungi are also assumed to play a significant role in structuring plant communities and increasing their productivity (van der Heijden *et al.* 1998). The results of the present work suggest that protozoa may play an important role in increasing plant nutrition and structuring plant communities *via* interactions with AM fungi which so far has not been considered. Moreover, it needs to be investigated whether interactions of mycorrhizal fungi with other protozoa species/groups function in the same way. Soil animal food webs are assumed to essentially rely on root derived C (Pollierer *et al.* 2007). This conclusion is supported by my observations that hyphae of AM fungi drain and translocate plant C into nutrient patches forming hotspots for microbial and invertebrate decomposers. From the plant perspective, C allocation towards symbionts

is essential to fuel plant-microbial interactions. Whether it is regulated at the whole plant level (systemic response to symbionts) or locally at the root level (according to the specific symbiotic traits of the root and its rhizosphere) needs further investigation. This can be achieved by using split-root systems for local or systemic inoculation with protozoa or AM fungi. As an example, a recent study (using non invasive ^{11}C labelling of plants in split root systems) revealed that antagonistic and beneficial symbionts have similar C sink strength. Moreover, C allocation in plants was systemically modulated by both antagonists and symbionts (Henkes 2008).

Soil food web interactions, such as the microbial loop in soil, drive nutrient cycling. In this thesis I showed that symbionts of different trophic affiliation are intimately connected and complement each other to decrease plant N shortage. Tight coupling of mobilization and uptake of nutrients is necessary for efficient fertilizer use and to increase crop yield. Management practices, such as crop rotation and ploughing, need to be adapted to avoid negative side effects on the soil food web to prevent loosing symbiotic partners and disrupt positive synergistic interactions for nutrient uptake, e.g. *via* mycorrhizal hyphae. More details on the functional role of specific symbionts are needed to predict effects on specific root symbionts, and thereby on plant nutrient acquisition and plant performance. Results of this thesis confirmed that the quality of organic matter in soils plays a crucial role in shaping the community structure of decomposers and this feeds back to plant biomass and plant C partitioning. For fostering interactions in belowground food webs to the benefit of plants, organic residue management systems, such as green manuring, need to be revisited in the context of multitrophic interactions.

Our model system allowed detailed insights into complex interactions of plants with their biotic root environment and provided information applicable for end users (see above). Hereafter, I will discuss limits of this approach and provide perspectives for future experiments.

In order to study plant–mycorrhiza-amoebae interactions, symbiont free treatments are necessary. A recently developed method reduces side effects to establish mycorrhiza free soil (Endlweber and Scheu 2006). However, so far no alternative method to autoclaving or gamma irradiation is available which allow destroying protozoa cysts in soil. Both methods exert strong side effects on soils, e.g. by increasing nutrient availability and releasing toxic compounds into the soil (Alphei and Scheu 1993). To reduce both, we washed the soil prior autoclaving it a second time. For re-establishing a microbial community resembling that in the field, we did not use a mixture of cultivable bacteria since cultivable bacteria presumably represent less than 3 % of the total bacteria species in soil. Hence, using this approach microbial groups necessary

for the functioning of the microbial loop in soil might be missing. Rather, we used a filtrate from fresh rhizosphere soil free of protozoa. Filtration also may have reduced microbial diversity, however, as proved by Rosenberg (2008) the inoculum procedure indeed allows to establish microbial communities resembling those in the field.

By applying stable isotope labelling techniques we linked plant C investment to N mobilization, i.e. the mineralization of nutrients from litter resources in soil. For tracing C and N fluxes we have chosen ^{13}C pulse labelling of shoots in combination with the addition of ^{15}N labelled litter. Pulse labelling of plant shoots and stable isotope probing in PLFAs, allowed investigating C allocation below the ground into free living root symbionts such as bacteria and into root associated symbionts such as AM fungi. However, recently fixed C represents only part of total plant C and thus our data do not allow calculating total plant C budgets. Moreover, C distribution at one point during plant development cannot be extrapolated in a straightforward way to other development stages (Kuzyakov and Domanski 2000). To investigate the dynamics of plant C fluxes series of pulse-labelling can be applied to determine changes in C allocation in response to changing source-sink relationships imposed by plant phenology (Robin 2006). Temporal changes indeed are crucial for understanding plant C and N fluxes. We harvested the plants once after 3 weeks. At this time our model plant *(P. lanceolata)* started to develop flowers, i.e. existed in a developmental stage which is characterized by shifts in N and C allocation towards reproductive structures and consequently reduced C supply to roots. Therefore, the activity of rhizosphere biota and plant nutrient uptake also likely were reduced (Marschner 1995). Thus, our "one-harvesting-point-approach" presumably underestimated the role of the studied rhizosphere interactions for plant nutrient acquisition and growth.

To complement our isotope approach, microbial methods such as reporter genes need to applied to obtain detailed information on the functioning of the microbial community. C fluxes into soil and shifts in the C mineralizing community can be assessed *via* rhizobacteria marked with *lux* and *gfp* reporters (Killham and Yeomans 2001). Additionally, functional gene analysis may allow to investigate the structure and functioning of nitrifying and denitrifying microorganisms (Patra *et al.* 2006). Finally, plant mutants may be used to separate hyphal and root pathways for nutrient uptake and signalling in protozoa-AM fungi-plant interactions. The complementary use of these new tools may allow obtaining a mechanistic understanding of the role of protozoa in plant N and P nutrition.

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Declaration

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The work presented in this thesis is the result of original research carried out by myself, whilst enrolled in the Institute National Polytechnique de Lorraine and the Technische Universität Darmstadt. This work was conducted independently and has not been submitted for any other degree of award in any other university or educational establishment.

Robert Koller

September 2008

INSTITUT NATIONAL
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AUTORISATION DE SOUTENANCE DE THESE
DU DOCTORAT DE L'INSTITUT NATIONAL
POLYTECHNIQUE DE LORRAINE

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VU LES RAPPORTS ETABLIS PAR :
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à soutenir devant un jury de l'INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE, une thèse intitulée :

"Amoebae in the rhizosphere and their interactions with arbuscular mycorrhizal fungi : effects on assimilate partitioning and nitrogen availability for plants"

en vue de l'obtention du titre de :

DOCTEUR DE L'INSTITUT NATIONAL POLYTECHNIQUE DE LORRAINE

Spécialité : « **Sciences agronomiques** »

Fait à Vandoeuvre, le 27 octobre 2008
Le Président de l'I.N.P.L.,
F. LAURENT

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Nancy, 11/2008

… und abschalten!

(Peter Lustig)