COMMON ARBUSCULAR MYCORRHIZAL NETWORKS:

TRADE OF CARBON AND SOIL NUTRIENTS BETWEEN DIFFERENT PLANT SPECIES AND THEIR SHARED FUNGAL SYMBIONT

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Dekan

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..... **III**

SUMMARY ..... **V**

1 GENERAL INTRODUCTION **1**

1.1 MYCORRHIZAL SYMBIOSIS 1

1.2 ARBUSCULAR MYCORRHIZA 4

1.3 RESOURCE EXCHANGE IN THE AM SYMBIOSIS 12

1.4 PLANT-FUNGAL INTERACTION 19

1.5 ROLE AND FUNCTION OF AMF IN PLANT COMMUNITIES 22

1.6 PHOSPHORUS ACQUISITION BY PLANTS 29

1.7 AIMS OF THE THESIS 41

2 MYCORRHIZAL NETWORKS: COMMON GOODS OF PLANTS SHARED UNDER UNEQUAL TERMS OF TRADE **43**

2.1 ABSTRACT 44

2.2 INTRODUCTION 45

2.3 RESULTS 48

2.4 DISCUSSION 55

2.5 CONCLUSION 58

2.6 MATERIAL AND METHODS 59

2.7 SUPPLEMENTAL MATERIAL 63

3 EXPRESSION OF PHOSPHATE TRANSPORTER GENES IN *SORGHUM* AND *LINUM*: DEVELOPMENTAL ASPECTS AND REGULATION IN A COMMON MYCORRHIZAL NETWORK **67**

3.1 ABSTRACT 68

3.2 INTRODUCTION 69

3.3 MATERIALS AND METHODS 71

3.4 RESULTS 76

3.5 DISCUSSION 84

3.6 SUPPLEMENTAL MATERIAL 89

4 CARBON AND NITROGEN METABOLISM IN MYCORRHIZAL NETWORKS AND MYCOHETEROTROPHIC PLANTS OF TROPICAL FORESTS: A STABLE ISOTOPE ANALYSIS 97

4.1 ABSTRACT 98

4.2 INTRODUCTION 99

4.3 RESULTS AND DISCUSSION 102

4.4 CONCLUSION 112

4.5 MATERIALS AND METHODS 113

4.6 SUPPLEMENTAL MATERIAL 117

5 TRACKING THE CARBON SOURCE OF ARBUSCULAR MYCORRHIZAL FUNGI COLONIZING C_3 AND C_4 PLANTS USING STABLE CARBON ISOTOPE RATIOS 123

5.1 ABSTRACT 124

5.2 SHORT COMMUNICATION 125

6 GENERAL DISCUSSION 131

6.1 DIVERSITY OF PLANT INTERACTIONS 131

6.2 TIT FOR TAT? 133

6.3 RESOURCE EXCHANGE AT PLANT-FUNGAL INTERFACE 135

6.4 REPRODUCIBILITY OF THE FUNCTIONAL EXPERIMENT 138

6.5 PERSPECTIVES 139

6.6 CONCLUDING REMARKS 142

7 APPENDIX 145

8 REFERENCES 155

9 CURRICULUM VITEAE 183

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SUMMARY

Plants commonly live in symbiotic associations with arbuscular mycorrhizal fungi (AMF). They invest substantial amounts of photosynthetic products to feed their fungal partners, which, in return, provide mineral nutrients foraged in the soil by their hyphal networks. AMF may supply up to 90 % of the host plant's nitrogen (N) and phosphorus (N) requirements. Moreover, AMF are important determinants of plant community structure and ecosystem productivity. Typically, AMF exhibit little host-specificity; a single individual may simultaneously colonize multiple plants, even from different species and thereby it forms far-reaching common mycorrhizal networks (CMNs). Hence, the interconnected plants share their C investments and nutritional benefits of the common fungal partner. This fact arises the question about the terms of trade between plants and their shared fungal partners. Or in other words, what is the C investment of a given plant into a CMN, and what is the return of this investment in terms of mineral nutrients provided by the CMN? However, up to now, the relationship between carbon investment and nutritional benefit of different plants engaged in a CMN has never been assessed.

To address the terms of trade in a CMN experimentally, we set up microcosms containing a pair of test plants, interlinked by a CMN of *Glomus intraradices* or *G. mosseae*. The plants were flax (*Linum usitatissimum*) and sorghum (*Sorghum bicolor*) grown either in "monocultures", as pair of identical plant species, or in a "mixed culture", as pair of different plant species. The microcosms were compartmented by nylon mesh screens to separate the CMN physically and functionally from the plant roots. Flax (a C_3-plant) and sorghum (a C_4-plant) display distinct C isotope compositions. This allowed us to differentially assess the C investment of the two plants into the CMN through the analysis of the C isotopic signature of isolated AMF hyphae or spores, or, with higher precision, of the AMF-specific fatty acid C16:1ω5. In parallel, we determined the plants' return of investment by measuring the acquisition of nutrients *via* CMN, using ^{15}N and ^{33}P as tracers only accessible for AMF hyphae. Plant growth response was determined by assessing the biomass of the plants.

Interestingly, we found a strong asymmetry in the terms of trade: when the CMN was formed by *G. intraradices,* flax invested only little C but gained up to 94 % of the CMN-mediated N and P while sorghum invested massive amounts of C without receiving a corresponding nutritional gain. The acquisition of nutrients was more balanced with a CMN formed by *G. mosseae*. However, sorghum still contributed the lion's share of C to the CMN. Nonetheless, in both cases sorghum was barely affected in growth, probably because it had a surplus of C. Excitingly, the growth of flax was highly increased due to the facilitated nutrient uptake *via* the CMN, which increased the overall biomass production in the mixed culture compared to the mean of the monocultures.

Many mycorrhizal plants are highly dependent on AMF for P acquisition; moreover the mycorrhizal P uptake usually dominates the plant's P acquisition. The mycorrhizal P uptake pathway starts in the soil far away from the roots, where AMF hyphae forage for immobile inorganic phosphate (P_i). The AMF hyphae take up P_i and translocate it to the roots. Inside the root, P_i is transferred from fungus to plant with the help of specific P_i transporters induced by the AMF. Remarkably, these AM-inducible P_i transporters are crucial for symbiotic P_i acquisition. In order to characterize the plants P acquisition *via* the CMN in our model system, we described for the first time P_i transporter genes belonging to the *Pht1* gene family in flax and sorghum. We found that the expression of these P_i transporter genes was highly dependent on the presence and identity of the AMF. Surprisingly, the plant's mycorrhizal P_i uptake appeared to be independent of the expression levels of AM-inducible P_i transporters in the roots. The genes showed very similar expression levels, even if the P_i uptake was dramatically different between the treatments. Nevertheless, AM-inducible P_i transporters showed different expression levels depending on culture system indicating that interconnected plants can influence the neighboring plant's gene expression.

An extreme example of terms of trade in CMNs displays achlorophyllous mycoheterotrophic (MH) plants. Most MH plants obtain, besides nutrients, their entire C from CMNs and thus indirectly exploit neighboring autotrophic plants. While temperate MH plants associated to ectomycorrhizal fungi are well described, tropical MH plants often associated to AMF are overlooked due to difficulties of examining AMF tissue. By analyzing AMF spores, we were able to investigate C and N isotopic signature of MH plants, green plants and AMF in Caribbean rainforests. These organisms displayed

similar C and N isotopic signatures, while temperate MH plants, fungi and green plants differ in their isotopic signature, suggesting differences in C and N exchange between the two MH systems. Furthermore, the isotopic analysis revealed canopy trees as main resource provider for AMF and MH plants in the Caribbean forests. Thus, we provide a first description of the autotrophic – AMF – MH continuum in tropical forests.

In order to track the C source of the CMN in our model system, we used different methods to make use of the C isotopic signature of AMF. In the last part of this thesis, we compare three methods to analyze the C isotopic signature in the AMF. Bulk C isotope analysis of washed extraradical mycelium is possible, but has the drawback of potential contamination from non-mycorrhizal organic sources. Bulk C isotope analysis of isolated AMF spores yields more reliable results but is rather tedious and most applicable for field studies. Therefore, we explain, in detail, a more refined analysis based on the extraction of lipids from soil, followed by analysis of the AMF biomarker fatty acid C16:1ω5.

In summary, this PhD thesis describes for the first time terms of trade in a CMN shared by two plants. The nutritional return provided by the fungus differed greatly between the examined plants and was not related to the extent of C investment, but dependent on the involved AMF species. However, the huge differences in nutrient uptake were not reflected in the expression levels of AM-inducible P_i transporters. In MH plants the use of the CMN is not only asymmetric, but even unidirectional. The investigation of both systems, MH and mixed culture, revealed that plant growth can be promoted by asymmetric use of CMNs. We propose that thanks to an exchange of surplus resources this can occur without impairment of the donor plant. Finally, the herein described mechanisms may help to understand the great impact of AMF on plant community structure and productivity.

1 GENERAL INTRODUCTION

1.1 MYCORRHIZAL SYMBIOSIS

In terrestrial ecosystems, almost all life is directly or indirectly reliant on the primary production by plants (Begon et al. 1996). They produce organic compounds by fixing atmospheric carbon (C) dioxide in the process of photosynthesis using light as source of energy. Thus, plants are autotrophic, meaning self-feeding organisms. In contrast, most of bacteria, fungi and animals are not able to produce organic compounds out of simple molecules and are therefore heterotrophic organisms depending on the plants' C. However, the growth of plants is not only based on atmospheric C allocation, but also strongly dependent on soil resources, namely mineral nutrients and water. Intriguingly, the majority of terrestrial plant species forages the rhizosphere, the narrow zone of soil surrounding roots, *via* symbiotic associations with fungi. This symbiosis of plants and fungi is known as mycorrhiza, which literally means "fungus-roots". The term symbiosis was strongly coined by the work of de Bary (1887) and denotes a close living together of dissimilar organisms ranging from parasitic to mutualistic interactions. The core process of the mycorrhizal symbiosis is the exchange of C photosynthetically fixed by the plant and nutrients collected in the soil by the hyphal network of the fungi. For plants associated to mycorrhizal fungi, mycorrhizae are even believed to be the main organ responsible for nutrient acquisition (van der Heijden et al. 2008). Therefore, they are one of the most important symbioses in terrestrial ecosystems (Smith and Read 2008). Moreover, it is supposed that the mycorrhizal symbiosis assisted plants to conquer the harsh terrestrial environment (Pirozynski and Malloch 1975).

Different types of mycorrhiza

Mycorrhizae are formed by fungi from different taxa (Zygomycota, Glomeromycota, Ascomycota and Basidiomycota) and by plants from an even wider range, including mosses (Anthocero-phyta, Marchantiophyta and Bryophyta), ferns (Pteridophyta) and higher plants (Spermato-phyta). The mycorrhizal symbioses are described in detail in the book of Smith and Read (2008). A common classification divides the mycorrhizae in several groups according to different structural characteristics and fungal taxa. An overview of different types of mycorrhizae is given in Table 1.1. However, there are some common characteristics applying to all types of mycorrhiza: (i) penetration of plant roots by hyphae, (ii) hyphal growth in the root cortex, (iii) formation of structures for resource exchange, and (iv) extensive proliferation in the external phase to scan the soil for nutrients and new host plants.

Most mycorrhizal fungi, as arbuscular, ericoid and orchid mycorrhizal fungi penetrate plant cells and form intracellular structures. An exception is built by the ectomycorrhizae, which form, instead of intracellular structures, very dense hyphal networks enveloping epidermal and cortical root cells and build thereby the so-called Hartig net (Bonfante and Anca 2009).Arbuscular mycorrhizae are widespread among various plant taxa (Brundrett 2009), while ericoid and orchid mycorrhizae are restricted to the order Ericales and the family Orchidaceae, respectively (Smith and Read 2008). Ectomycorrhizae are characteristic for trees and shrubs, and are only rarely formed by herbs (Bonfante and Anca 2009). Remarkably, several species of both plants and fungi are able to form different types of mycorrhiza, depending on the counterpart of the symbiotic association (Wang and Qiu 2006, Smith and Read 2008). For instance, trees of the genera *Salix* and *Populus* can be colonized by arbuscular and ectomycorrhizal fungi (Walker and McNabb 1984, Dhillion 1994). So far, however, not much is known about the interplay and functional complementarity of arbuscular and ectomycorrhizal symbiosis when a single plant simultaneously forms both types. Nevertheless, arbuscular mycorrhiza is by the far the most abundant mycorrhizal type, followed by orchid and ectomycorrhiza (Brundrett 2009).

Table 1.1 Characteristics of different mycorrhizal types.

Types of mycorrhiza	**Arbuscular**	**Ecto**	**Ericoid**	**Orchid**
Fungal taxa	Glomeromycota	Basidio- and Ascomycota	Ascomycota	Basidiomycota
Plant taxa	Bryophyta, Pteridophyta, Gymnospermae, Angiospermae	Gymnospermae, Angiospermae	Ericales, Bryophyta	Orchidales
Fungi +/- septae	-	+	+	+
Intracellular structures	+	-	+	+
Hartig net	-	+	-	-

Modified after Smith and Read (2008).

Functional role and global distribution

Different mycorrhizal types also differ in their functional role and global distribution. Ericoid and ectomycorrhizal fungi have saprophytic capabilities and are able to access nutrients sequestered in complex organic polymers (Read and Perez-Moreno 2003). Therefore, ericoid and ectomycorrhiza are predominant in ecosystems with highly organic soils such as heathlands and boreal forests, respectively. But also in temperate forest, with enhanced nutrient mineralization, ectomycorrhiza is the predominate mycorrhizal type (Courty et al. 2010). Arbuscular mycorrhiza plays a crucial role in ecosystems with soils exhibiting mainly mineral nutrients and low phosphorus (P) to nitrogen (N) ratios hence decreasing P availability (Read and Perez-Moreno 2003). Anyhow, with increasing latitude and altitude the importance of arbuscular mycorrhiza is decreasing on the expense of ecto- and ericoid mycorrhiza (Read and Perez-Moreno 2003). Generally speaking, AM are characteristically found in species-rich ecosystems, in contrast to ectomycorrhizae which predominate in forest ecosystems where only a few host species are present (Smith and Read 2008).

1.2 ARBUSCULAR MYCORRHIZA

Introduction

Arbuscular mycorrhiza (AM) is the most abundant mycorrhizal type and is probably even the most widespread terrestrial symbiosis (Parniske 2008). AM is formed between the majority of land plants and soil fungi belonging to the phylum Glomeromycota (Schüssler et al. 2001). The name arbuscular is derived from the characteristic structure, the arbuscule, which is formed in the cortical cells of roots colonized by arbuscular mycorrhizal fungi (AMF; Smith and Read 2008). AMF live in two distinct sections; (i) the intraradical mycelium in the roots by which C and nutrients are exchanged with the plant and (ii) the extraradical mycelium in the soil which absorbs immobile nutrients (Figure 1.1; (Smith et al. 2011). This symbiosis is perhaps just as ancient as the origin of land plants. Fossil spores and symbiotic structures dating back to the Ordovician (460 million years ago) support the hypothesis that AMF were the important fungal partner supporting the colonization of land by vascular plants (Remy et al. 1994, Redecker et al. 2000).

Arbuscular mycorrhizal fungi

AMF are obligate symbionts receiving all their C from the associated plants (Smith and Read 2008). They belong exclusively to the phylum Glomeromycota (Schüssler et al. 2001), which consists only of fungi that are generally considered to live in symbiotic associations. AMF are harbored in the order Glomerales including the families Glomeraceae and Claroideogleraceae; the order Diversisporales including the families Gigasporaceae, Acaulospraceae, Entrospora-ceae, Pacisporaceae and Diversisporaceae; and the order Paraglomerales including the family Paraglomeraceae (Schüssler and Walker 2010). The order Archaeosporales harbors besides AMF in the families Ambisporaceae and Archeosporaceae, also a fungal species who has a cyanobacterium as symbiotic partner - namely *Geosiphon pyriforme* of the family Geosiphona-ceae (Schüssler et al. 1994, Schüssler and Walker 2010).

Remarkably, AMF appear to be strictly asexual, as so far no evidence for sexual propagation could be found (Smith and Read 2008, Sanders and Croll 2010). Nevertheless, it is still possible that AMF have a cryptic sexual reproduction that has not yet been captured with the current methods (Kück and Pöggeler 2009). However, the seemingly asexual nature of the AMF entails the problem that the "Biological Species Concept" – which implies that two organisms belong to the same species if they are able to produce fertile offspring - is not applicable to define AMF species. Therefore, this is commonly done on the basis of different spore morphologies (Smith and Read 2008). The bare number of species (150-200) in the Glomeromycota underestimates the diversity in this phylum, apparent in the huge phenotypic and genetic diversity found within an single AMF species (Koch et al. 2004, Munkvold et al. 2004). AMF are not only asexual but also haploid. Nevertheless, the genetic diversity is very high, even in single isolates. Thus, several different sequences of nuclear ribosomal DNA, different AFLP patterns and even different versions of certain genes can be found within one strain (Rosendahl and Taylor 1997, Clapp et al. 2001, Kuhn et al. 2001, Pawlowska and Taylor 2004). How exactly the different sequences are organized is a still unsolved question and is of considerable interest for revealing the rules of gene flow to the next generation. There are two theories under discussion described in Pawlowska and Taylor (2004); (i) the heterocaryotic model, where the nuclei are assumed to be genetically different, or (ii) the homocaryotic model which proposes identical nuclei each containing the total genetic diversity.

Genetic exchange between closely related AMF individuals might occur by anastomosis (Croll et al. 2009, Sbrana et al. 2011). Anastomoses are fusions between hyphae establishing a cytoplasmic continuity and allowing nuclear migration (Giovannetti et al. 2004). Furthermore, a recent study discovered a set of meiosis-specific genes suggesting that this process could occur in AMF (Halary et al. 2011). However, the importance of the meiosis machinery in AMF has not been shown so far, although it is considered as hallmark of sexual reproduction in eukaryotes (Schurko et al. 2009).

Plants forming AM symbiosis

Plants of a broad range of taxa, including ferns and liverworts are associated to AMF and it can be assumed that about 200'000 or 70-90 % of all plants species are able to from AM symbiosis (Wang and Qiu 2006, Brundrett 2009). However, many species have been recorded to occur in both mycorrhizal and non-mycorrhizal states. The reasons for missing colonization of a potentially mycorrhizal species are for example the on-site lack of an appropriate AMF partner or environmental conditions such as high nutrient availability (Smith and Read 2008). Plant species, which are colonized only under certain conditions, are often defined as "facultative mycorrhizal", to distinguish them from "obligate mycorrhizal" species whose development appears to be dependent on the symbiotic association. AMF predominantly occur in herbaceous plants, but are also widely found in trees of tropical and temperate forests (Tawaraya et al. 2003, Wang and Qiu 2006). However, AMF might even have the ability to invade the roots of almost all terrestrial plants, because of their ancient origin at the beginning of terrestrial plant evolution (Brundrett 2002). Thus, ectomycorrhizal, ericoid and orchid mycorrhizal, as well as non-mycorrhizal species, evolved secondary during a later period of plant evolution and appear in lineages of more recent origin.

Several plant families are thought to be non-mycorrhizal, such as Chenopodiaceae, Brassicaceae, Caryophyllaceae, Polygonaceae, Juncaceae and Proteaceae (Wang and Qiu 2006, Brundrett 2009). Anyhow, the "non-mycorrhizal" state of a plant family is difficult to attest and is to a certain degree depending on the sample size of investigation. Hence, some families have both mycorrhizal and non-mycorrhizal members and even in predominantly non-mycorrhizal groups, some mycorrhizal species do occur. Furthermore, a generalization of the mycorrhizal state of a plant may be difficult, because it is highly dependent on environmental factors (Smith and Read 2008). Nevertheless, out of 3'350 tested plants of the family Brassicaceae not a single plant species has found to be mycorrhizal (Brundrett 2009). The Brassicaceae also include the most commonly used model organism in plant biology and genetics *Arabidopsis thaliana,* which even experimentally could not be forced to become mycorrhizal so far. This can be seen as a big disadvantage for molecular research on the AM symbiosis (Bonfante and Genre 2008). However, non-mycorrhizal plant families and species are distantly related to each other, indicating that the non-mycorrhizal state evolved separately several times

in plants (Brundrett 2002). It is thought that several factors may have led to loss of the mycorrhizal state, as for example adaption to growth in nutrient rich or disturbed environments and development of non-mycorrhizal nutrient-acquiring strategies (Pate 1994, Marschner 1995, Brundrett 2009). But the mechanisms by which the fungi are prevented from colonizing the plant root are not known so far (Smith and Read 2008).

Establishment of the AM symbiosis

AMF colonization of roots can be initiated by hyphae growing from spores or previously colonized roots (Figure 1.1). Spores are thick-walled propagules of AMF that can be distributed by zoochory and persist for many years in the soil (Smith and Read 2008). They can germinate also in absence of plants, but to complete their life cycle, the obligate fungal symbiont needs the C from the autotrophic partner (Parniske 2008). Therefore, it is mandatory for AMF to maximize the chance of encountering a plant root before depletion of the C resource of the spore, which can be facilitated by diffusible signals of both partners (Bonfante and Requena 2011). In analogy with the Nod factors produced by symbiotic rhizobial bacteria fixing nitrogen in nodules, the AMF diffusible molecules were named Myc factors. The existence of such Myc factors was relatively early indicated by activation of a symbiosis related gene (Kosuta et al. 2003), and later by affecting signal transduction and root branching due to diffusible molecules (Kuhn et al. 2010, Mukherjee and Ane 2011). However, the structure of these Myc factors has only recently been identified as simple lipochitooligosaccharides, which are very similar to Nod factors (Maillet et al. 2011). Remarkably, it has been shown that the process of AM and rhizobial symbiosis formation uses a partially overlapping signaling route in the plant, the common symbiosis (SYM) pathway (Catoira et al. 2000). In the SYM pathway, microbial signals are transduced to a calcium signal that activates symbiosis essential genes (Oldroyd and Downie 2006). Anyhow, the initial and the last step of the SYM pathway differ for the fungal and bacterial symbiont (Bonfante and Requena 2011).

Besides the AMF also the potential plant partners excrete diffusible signals. The stimulatory effect of plant root exudates on AMF has been recognized for a long time, but the molecular identity of the “branching factors” has relatively recently been identified (Parniske 2008). Strigolactones were found to be responsible for the induction of branching and alterations in fungal physiology and mitochondrial activity (Akiyama et al.

2005, Besserer et al. 2006). This class of compounds can also stimulate spore germination in some AMF (Bouwmeester et al. 2007). Interestingly, strigolactones were identified at first as a potent germination inducer of seeds of the parasitic plant genus *Striga* and other parasitic plant genera (Cardoso et al. 2011). Moreover, strigolactones were recently identified as novel endogenous plant hormones in diverse angiosperms (Gomez-Roldan et al. 2008, Kapulnik et al. 2011).

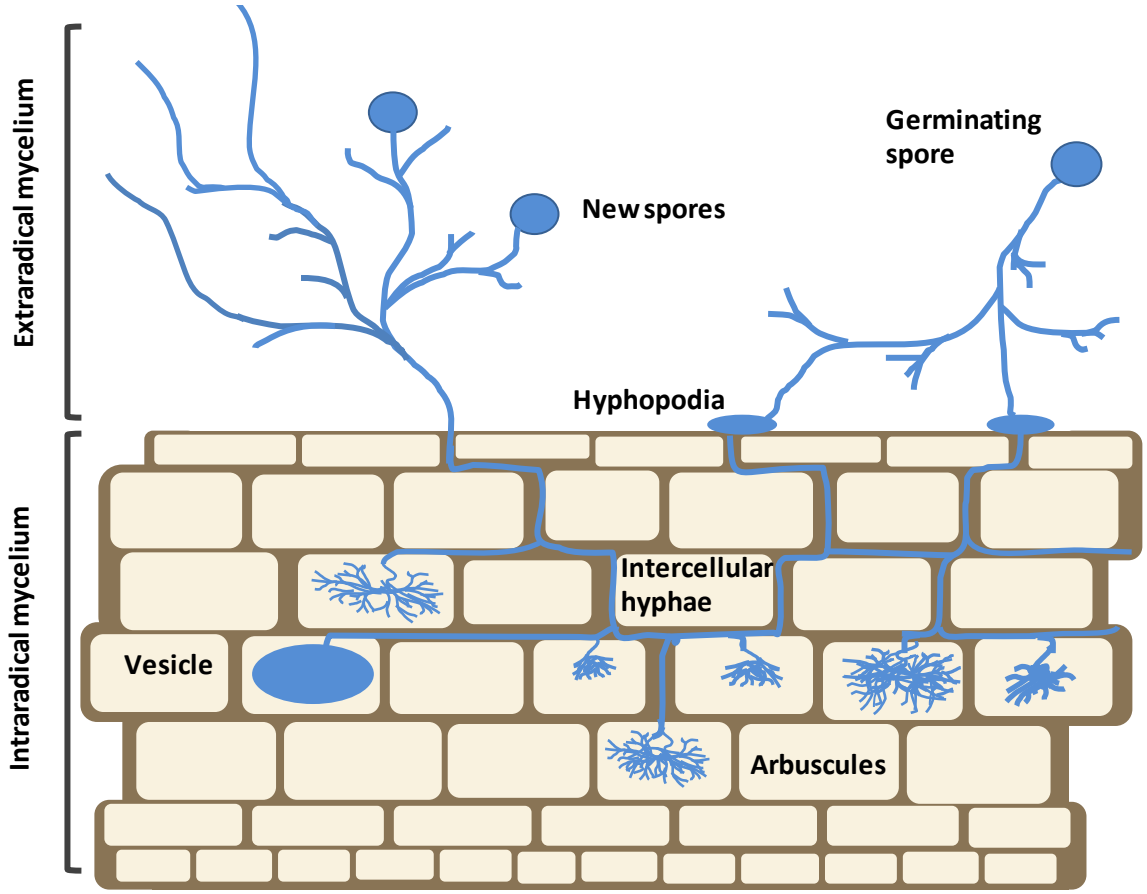

Figure 1.1 Arum type structures of AMF in plant roots and soil. Growth of AMF hyphae is stimulated by root exudates from plants. In contact with the root, the hyphae form hyphopodia to penetrate epidermal root cells and proliferate intercellularly to the inner cortical cell layer. The intraradical hyphae penetrate the cortical cells and form arbuscules and vesicles. After establishing the intraradical mycelium the AMF exits the root and forms the extraradical mycelium comprised of hyphae foraging the soil for nutrients and spores.

As soon as direct contact between the symbionts is established, the AMF attaches to the plant root by forming a hyphopodium, a swollen hypha that pegs the root epidermis and thereby enables intracellular colonization (Figure 1.1; Bonfante and Genre 2008). Subsequently, fungal hyphae penetrate the epidermal cell, which is supported by the plant by developing a penetration apparatus through which hyphae grow (Genre et al. 2005, Genre et al. 2008). Inside the root, the intraradical mycelium proliferates in two different morphological growth patterns (Smith and Read 2008): (i) the *Arum* type is characterized by intercellular growing hyphae penetrating cortical cells by side branches, in which arbuscules are formed (Figure 1.1). Arbuscules are highly branched structures, which are considered to be the key element for symbiotic nutrient exchange similar to haustoria of pathogenic fungi. (ii) The *Paris* type, in contrast, is characterized by intracellular growth of hyphae from cell to cell, in which coils are formed. Remarkably, both plant and fungal partners determine the mycorrhizal type, and it has been shown that arbuscules and hyphal coils are involved in nutrient transfer (Cavagnaro et al. 2001, Dickson 2004, Dickson et al. 2007). The intracellular structures of the fungus are completely enveloped by the plant plasma membrane, the so-called perisymbiotic or peri-arbuscular membrane, and thus remain within the apoplast of the plant cell without direct contact to the symplasm (Figure 1.2; Smith and Read 2008). Albeit the periarbuscular membrane is a continuation of the plasma membrane, it is highly differentiated and equipped with symbiosis-specific trasnpoters (Pumplin and Harrison 2009). Moreover, the plant-fungal interface is separated by the periarbuscular space, an apoplastic region framed by the periarbsucluar membrane of the plant and a specialized membrane of the fungus (Figure 1.2). This organization of two facing plasma membranes is important with respect to the control potential of both partners in the symbiotic nutrient transfers (Smith and Smith 2011).

Besides arbuscules and hyphal coils, vesicles are the other prominent structure of the intraradical mycelium, but these are not formed by all AMF species (Figure 1.1). Similar to spores, vesicles are thick-walled structures containing high amount of lipids and function as storage organs and propagules (Smith and Read 2008).

After establishing the intraradical mycelium the AMF exits the root and forms the extraradical mycelium, the largest fungal organ, which can amount for up to 90% of total fungal biomass (Olsson et al. 1999). The mycelium consists of diffuse coenocytic

(aseptate) hyphae exhibiting two different morphologies and functions (Bago et al. 2004): (i) runner hyphae are fast-growing and have relatively wide diameters (up to 20 µm) exploring the soil radially for nutrient rich patches and for additional C sources in form of new roots. (ii) Other hyphae have narrow diameters (2 µm), are highly branched and specialized for acquiring nutrients in small soil pores. The life spans of the thicker hyphal structures appear to be longer and to determine the development of the extraradical mycelium, whilst the thinner hyphae have a half-life of days (Staddon et al. 2003). The extraradical mycelium has quite diverse morphological characteristics depending on the AMF species, as for example hyphal diameters and distance of growth away from the root to absorb nutrients (up to 25 cm; Jakobsen et al. 1992, Drew et al. 2003, Jansa et al. 2003). The density of hyphae in soil can be used to estimate the extent of the extraradical mycelium. Hyphal length densities in pot experiments are variable and usually in the range of 1–40 meter hyphae per gram of soil (m g^{-1}) depending on the identity of both symbiotic partners (Munkvold et al. 2004, Smith et al. 2004). In field, hyphal length densities can even reach up to 100 m g^{-1} (Miller et al. 1995). Generally, the density of extraradical hyphae is much higher than that of roots exhibiting an advantage in nutrient acquisition (Li et al. 2008). Furthermore, the dense extraradical network forms the major part of total soil biomass (Olsson et al. 1999) and contributes substantially to the stabilization of soil by agglomerating soil particles (Rillig and Mummey 2006).

The life cycle of AMF is completed by the anew production of spores, which are mainly produced as single spores at the end of branching hyphae of the extraradical mycelium (Figure 1.1). However, in certain AMF, spores can be found in clusters, the so-called sporocarps, and in other AMF species (e.g. *Glomus intraradices*) spores can be formed even within the roots (Smith and Read 2008).

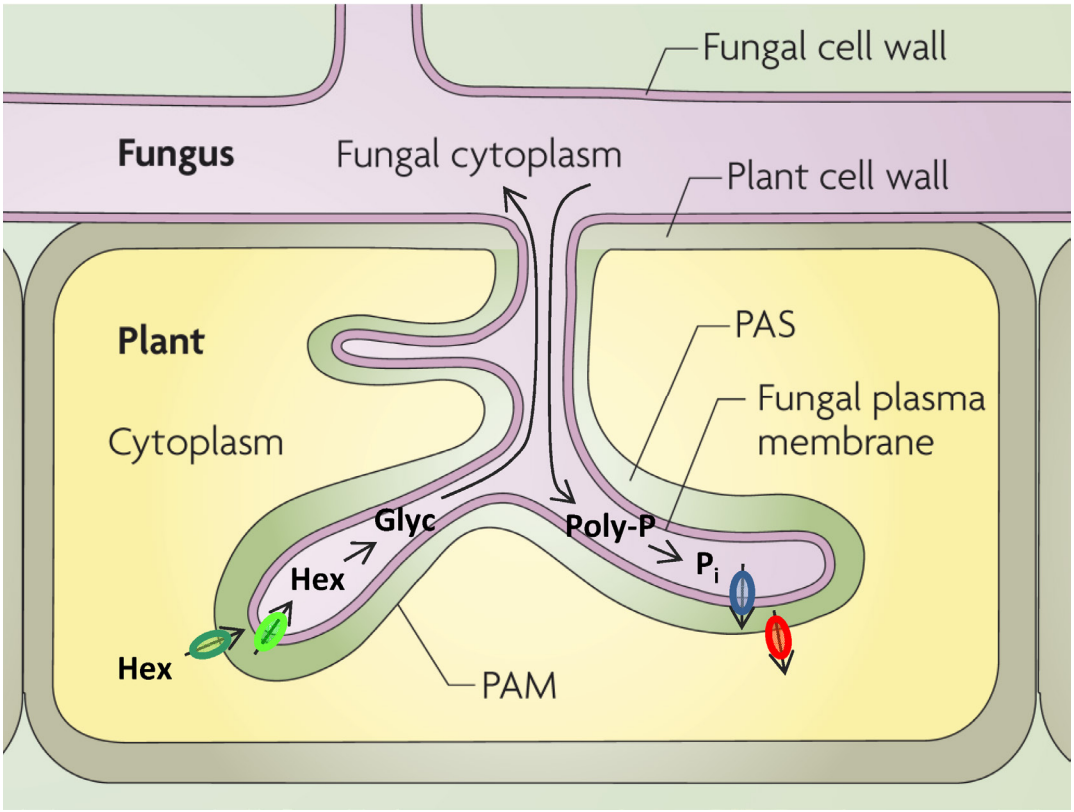

Figure 1.2 Schematic drawing of the plant fungal-interface of arbuscular mycorrhiza (AM). Each fungal arbuscule within a plant cell is surrounded by a plant-derived periarbuscular membrane (PAM) that excludes the fungus from the plant cytoplasm. The apoplastic interface between the fungal plasma membrane and the plant-derived PAM is called the periarbuscular space (PAS). The plant releases carbon (C) into the PAS *via* monosaccharide transporters (dark green circle) in form of hexoses (Hex), which is taken up by fungus acorss the fungal plasma membrane *via* fungal monosaccharide transporters (light green cricle). Within the fungal cytoplasm, hexoses are converted into glycogen (Glyc) and lipids and are transported to the extraradical mycelium. In return, nutrients that are acquired by the fungus from soil are delivered to the plant cell. Phosphate is transported towards the intraradical mycelium in form of poly phosphates (Poly-P), which is hydrolyzed in the arbuscules to P_i. P_i transporters of the fungus (blue circle) release P_i in the PAS from where it gets translocated across the PAM *via* AM-inducible P_i transporter of the plant (red circle). Modified after Parniske (2008).

1.3 RESOURCE EXCHANGE IN THE AM SYMBIOSIS

Carbon nutrition

AMF have no saprophytic capabilities and therefore depend entirely on photosynthetically fixed C of their autotrophic symbiotic partners to finish their life cycle (Smith and Read 2008). Indeed, AMF consume up to 20% of plants photosynthates (Jakobsen and Rosendahl 1990, Bryla and Eissenstat 2005). Most of the allocated C is respired, but 25% can be found in the extraradical mycelium (Jakobsen and Rosendahl 1990). However, plants can at least partially control the substantial C allocation to the fungus indicated by decreased colonization levels and spore production, especially under high nutrient availability (Oehl et al. 2003). Interestingly, increased C cost by shading did not reduce the C flow to the fungus (Olsson et al. 2010). The transfer of C from plant to fungus occurs in form of hexoses, probably as glucose and fructose (Pfeffer et al. 1999). It is assumed that the plant shoot delivers sucrose to AM roots, where it gets hydrolyzed by plant enzymes. The so generated hexoses are then transferred to the fungus (Figure 1.2; Blee and Anderson 2002, Ravnskov et al. 2003). In agreement, it has been shown that the intraradical mycelium of AMF has a high capacity to absorb different types of hexoses, which get rapidly incorporated into trehalose and glycogen (Shachar-Hill et al. 1995, Solaiman and Saito 1997). Moreover, lipids, which constitute by far the larges C pool of the fungi, are synthesized in the intraradical mycelium before being translocated to extraradical structures (Pfeffer et al. 1999, Bago et al. 2003). However, the exact location where C transfer from plant to fungus takes place remains obscure (Hodge et al. 2010). There is a general assumption that arbuscules are involved in C exchange, but definitive evidence is still lacking. Additionally, it has been suggested that the interface between the intercellular hyphae and the root cortical cells could be of importance. Evidence that support the latter is given by the activity of an ATPase occurring at the intercellular hyphae (Gianinazzi-Pearson et al. 1991). However, the first glomeracean hexose transporter has been identified in the fungus *Geosiphon pyriforme* (Schüssler et al. 2006) and more recently, a versatile high-affinity monosaccharide transporter (MST2) from *Glomus* sp. was discovered (Helber et al. 2011). This monosaccharide transporter is highly induced in AM symbiosis and is expressed in both intercellular hyphae and in arbuscules. Moreover, MST2 appears to be even crucial for the AM symbiosis indicating an essential role in the C transfer from plant to fungus (Helber et al. 2011).

Nutrient acquisition in AM symbiosis

The role of AMF in nutrient uptake by plants was investigated more than any other aspect of the AM symbiosis. Furthermore, AMF have been largely seen as functional extensions of the plant root system. However, this is probably simplistic and describes a rather plant-centric view of the AM symbiosis. In contrast, a more myco-centric view takes also the nutritional needs of the fungus into account (Fitter et al. 2000).

Experiments elucidating the role of AMF in delivering nutrients to plants have been conducted in compartmented systems (Figure 1.3A). These systems are comprised of at least two compartments; a root-hyphal compartment populated by a plant root colonized by AMF, and a hyphal compartment containing isotopic tracers. The compartments are usually subdivided by mesh barriers with a mesh size of not more than 25 µm, which makes them penetrable for AMF hyphae but not for plant roots (Figure 1.3B). By the use of such systems the roots and hyphae can be separated not only physically but also functionally. In experiments using compartmented systems, it has been shown that AMF can provide almost 100% of total phosphorus (P) acquired by plants (Smith et al. 2003b, 2004; see also section 1.5). Besides P, AMF deliver other immobile nutrients such as nitrogen, potassium, zinc and copper in substantial amounts to the plant (Marschner and Dell 1994). Nutrient uptake by AMF appears to be more dependent on the extent of the extraradical mycelium than on the level of root colonization (Jakobsen et al. 1992, Munkvold et al. 2004, Jansa et al. 2005). Moreover, AMF species apparently have a certain degree of functional complementarity in nutrient acquisition showed by synergistic effects of dual AMF colonization of a plant (Jansa et al. 2008). Hence, plants can increase their AMF-mediated nutrient acquisition by simultaneously harboring several fungal symbionts. Synergy between the fungi can probably be explained on the basis of differences in nutrient uptake strategies as displayed in different hyphal length densities (Koide 2000, Jansa et al. 2003).

AMF efficiently acquire soil nutrients by proliferating hyphae rapidly in nutrient rich patches or in accumulations of organic matter (Joner and Jakobsen 1995, Hodge 2001, Cavagnaro et al. 2005). How the extraradical mycelium scans the environment for nutrient rich patches in order to intensify their network is still unknown (Hodge et al. 2010). AMF acquires nutrients from root-free compartments with remarkable effectiveness and hyphal uptake may be just as efficient as the uptake of roots and

hyphae together (Jakobsen et al. 2002). For both roots and AMF hyphae inorganic ions constitute the primary nutrient source (Marschner and Dell 1994), but AMF also appear to influence the acquisition of nutrients from organic sources. AMF rely on saprophytic microorganisms to decompose the organic matter and release inorganic nutrients (Hawkins et al. 2000, Whiteside et al. 2009). Nevertheless, due to the extensive proliferation into accumulations of organic matter, AMF can effectively compete against the soil fauna during periods of rapid mineralization of nutrients (Jakobsen et al. 2002). Reciprocally, the presence of AMF enhances the growth of the co-located microorganisms by providing additional C (Toljander et al. 2007, Herman et al. 2012). Hence, AMF can influence indirectly, but positively plants acquisition of organic nutrients.

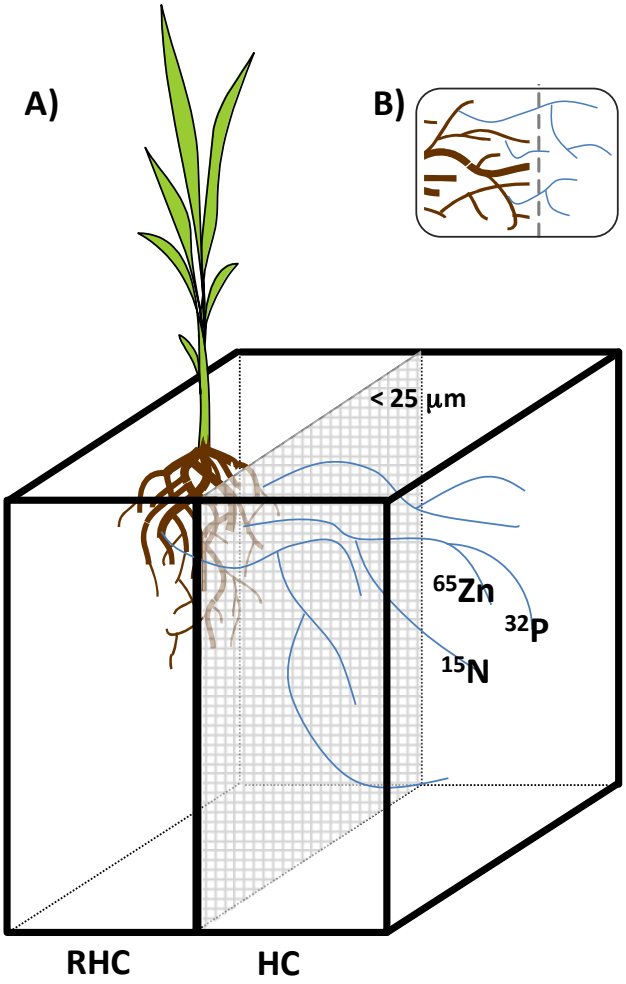

Figure 1.3 Schematization of a compartment system to study the role of arbuscular mycorrhizal fungi (AMF) in nutrient uptake of plants. (A) Compartment system consisting of two compartments subdivided by nylon mesh screens (mesh size <25 µm). The screen is pervious for AMF hyphae but not for roots and allow the separation into a root-hyphal-compartments (RHC) and a hyphal-compart-ment (HC). Thus, isotopic tracers such as ^{33}P for phosphorus, ^{15}N for nitrogen or ^{65}Zn for zinc supplied in the HC allow the analysis of AMF nutrient acquisition. (B) Small mesh size of the screen allows only AMF hyphae to penetrate, while plant roots are too thick to growth through.

Mycorrhizal nitrogen acquisition

Plants and AMF can absorb N in the two inorganic forms nitrate and ammonium from the soil (Marschner and Dell 1994). Both major inorganic N sources are regarded as relatively mobile in soil, nitrate to a higher extent than ammonium, and are therefore transported to roots by mass flow in the soil solution. Due to this rather high mobility of N, roots and hyphae are expected to have similar effectiveness in uptake of inorganic N (Marschner and Dell 1994, Atul-Nayyar et al. 2009). Anyway, in dry soils with reduced mass flow N supply of plants can be more locally restricted and therefore be more dependent on AMF (Tobar et al. 1994, Tinker and Nye 2000). Furthermore, organic N probably represents the largest portion of the total N pool in soil and the availability for plants and AMF is highly dependent on immobilization and mineralization by soil microorganisms (Hodge et al. 2000). In contrast, ecto- and ericoid mycorrhizal fungi are capable to access organic N sources directly and therefore are predominant in rather N limited ecosystems with organic soils (Read and Perez-Moreno 2003, Lambers et al. 2008).

Empirical data has shown that AM symbiosis only weakly impacts N nutrition in soil-grown plants (George et al. 1995, Hawkins et al. 2000, Reynolds et al. 2005). Besides the environmental factors discussed above, biological factors may help to explain the poor impact of AMF on plant N nutrition. AMF have a high N demand compared to plants apparent in their relatively low C to N ratio of 10:1 (Garraway and Evans 1984). In contrast, the C to N ratios of plants are ranging between 45:1 in leaves to 400:1 in woody stem (Sterner and Elser 2002). Thus, AMF need more N per unit biomass than plants and are strong competitors for N in the AM symbiosis. Indeed, when soil N is rare and is limiting the growth of plants and fungi, it can be assumed that the fungus will keep N to cover its own demands and will not deliver it to plant (Johnson 2010). Anyhow, increased N uptake has been observed in mycorrhizal legumes, but these findings have been attributed to the positive effects of AM-mediated P uptake on N_2 fixation (Smith and Read 2008).

Nevertheless, AMF have been shown to transfer considerable amounts of ammonium and nitrate to the plants in compartmented systems (Johansen et al. 1993, Johansen et al. 1996, Mäder et al. 2000). The N taken up by the fungus in form of ammonium is probably translocated within the hyphae as arginine, which is later on broken down to urea (Tian

et al. 2010). Ultimately the transfer to the plant occurs in form of ammonium again while the C skeleton remaining of arginine breakdown is retained in the fungus (Bago et al. 2001, Govindarajulu et al. 2005, Jin et al. 2005). Evidence for ammonium transfer has been given by the fact that plant ammonium transporters specifically involved in symbiotic N uptake have been identified in *Lotus japonicus* and *Medicago truncatula* and are showing preferential expression in arbuscular cells (Gomez et al. 2009, Guether et al. 2009). In the case of nitrate, the uptake *via* AMF is less well described so far. However, enhanced activities of nitrate reductase, glutamine synthetase, and glutamine synthase in the roots of mycorrhizal maize (*Zea mays*) indicate that the absorbed nitrate can be directly transferred to the root cells (Miransari 2011).

It has been assumed that AMF are unable to access organic N sources. Nevertheless, several studies examined the N uptake out of an organic pool by labeling experiments with ^{15}N and ^{13}C (Hodge 2001, Leigh et al. 2009). Plants were able to obtain, *via* hyphae of the associated AMF, up to 20 % of their total N from these organic patches. However, no transfer of ^{13}C could be detected indicating that AMF did not absorb organic N in intact form. These experiments display a further evidence, that AMF are not directly involved in the mobilization of organic N, but may increase the transfer of mineralized inorganic N of organic patches to the plant (Smith and Smith 2011).

Reciprocity of exchange

The core process of the AM symbiosis is the reciprocal exchange of resources; the fungus delivers soil nutrients to the plant and in return receives photosynthetically fixed C. Although the individual resource fluxes are well studied, their interdependence, or in other words the trade of carbon and mineral nutrients in the AM symbiosis is poorly understood. However, the trade volume is of substantial dimensions for both symbiotic partners, most apparent, in the obligate dependency of the fungus on plants C. But also the investment of up to a fifth of total assimilates implies the need for a sort of regulation for either site of the trade. Furthermore, in face of the evolutionary history and success of this partnership a regulation of the resource exchange between the two partners is expected (Kiers and Denison 2008). Such regulations should lead to reciprocal trade of resources in the AM symbiosis and could be achieved by favoring more beneficial associations. Additionally, symbiotic partners exploiting resources with little or no

beneficial returns, the so-called cheaters, should be negatively selected (Smith and Smith 1996, Egger and Hibbett 2004). Anyway, up to know not much is known about the linkage between investment and benefit in the AM symbiosis, but several models are under discussion.

A theoretical model describing a tight linkage between investments and beneficial return in the AM symbiosis is given by Fitter (2006). The mechanism proposed in this model is that resource exchange in the AM symbiosis is regulated by the fact that the delivery of nutrients across the plant-fungal interface directly stimulates the supply of C by the plant to a spatially defined location in the root. This mechanism would be resistant to colonization by non-beneficial fungi, if they fail to supply nutrients they will not stimulate an increased sugar supply. This model refers to the C allocation in non-mycorrhizal root systems, which responds to patchy distribution of nutrients in soil by specific and fine-scaled proliferation towards the nutrient sources (Drew 1975, Linkohr et al. 2002). It was assumed that plants are thereby able to favor the more beneficial fungal partners (Helgason and Fitter 2009). Indeed, preferential C allocation to the more beneficial AMF has been shown in a split root system harboring two different fungal species at each side (Bever et al. 2009). Accordingly, the C investment of the plant and the benefit received from the fungal partner would be tightly interlinked. Such a strict reciprocity between investments and benefits in the AM symbiosis has also been shown by recent studies comparing C allocation to P and N supply in root-organ cultures (Kiers et al. 2011, Fellbaum et al. 2012). They have shown that when different AMF species are simultaneously colonizing the same root, higher nutrient supply by the fungus was rewarded by higher C allocation. Moreover, positive relation between investment and benefit has also been found in other systems. Studies involving several plants have shown that plant indiviuals of different sizes obtain a benefit relative to their biomass (Kytoviita et al. 2003, Pietikainen and Kytoviita 2007).

Nevertheless, assuming a strictly reciprocal exchange of resources is probably too simplistic for the AM symbiosis, because it does not allow any cheating. Indeed, cheating is clearly occurring in case of mycoheterotrophic plant species exhibiting even unidirectional resource flows (Smith et al. 2009, Merckx et al. 2010; see section 1.5). Moreover, the model of a tight linkage in resource exchange can hardly explain the antagonistic AM associations seen experimentally, in which the C investment to the

fungus obviously is not recompensed in sense of beneficial returns (Johnson et al. 1997, Klironomos 2003). A novel hypothesis, based on a computer simulation model, postulates that mycorrhizal C and nutrient exchanges are not quantitatively interlinked, but are dependent on the symbionts' demands (Landis and Fraser 2008). In agreement, it has been proposed earlier that the AM symbiosis is based on the exchange of "luxury goods" (Kiers and van der Heijden 2006). The exchange of this surplus or luxury resources could be beneficial for both partners, if neither C for the plant nor mineral nutrients for the fungus are limited and therefore be evolutionary stable (Schwartz and Hoeksema 1998, Hoeksema and Bruna 2000). In this spirit, AM symbiosis can be seen as a "marketplace", on which symbionts offer their luxury resources and acquire the resources on demand. Hence, the amount of beneficial return would be rather dependent on the functional ability to gain resources of the symbiont than on the reciprocity of investment. This relationship could not only differ with different species pairs, but also for different life stages of the plants. Therefore, the investment in AMF may be important for plant survival, even if the benefit comes at a relative high expense and at a later point in time (van der Heijden and Horton 2009).

1.4 PLANT-FUNGAL INTERACTION

Plant response to AMF colonization

Different plant species show large differences in the response to AMF colonization. The different performances, including growth and nutrient status, between a mycorrhizal and a non-mycorrhizal plant is defined as mycorrhizal responsiveness (Jakobsen et al. 2002, Smith and Read 2008). An enormously wide range of mycorrhizal responsiveness has been reported, usually determined in experiments conducted in simplified systems comprising single plant-fungus relations under controlled conditions (Wilson and Hartnett 1998, Klironomos 2003, Tawaraya et al. 2003). The variation is impressively displayed in the study of Klironomos (2003) showing that in the 64 tested plant species all inoculated with the same AMF isolate the mycorrhizal growth response differed from -46 to +48 %. The outcome ranging from highly positive over neutral to negative was defined as the mutualistic-antagonistic continuum in the AM symbiosis (Johnson et al. 1997). However, the result of such simplified experiments should not be seen as unalterable. Thus, antagonistic plant-fungus combinations could become mutualistic on a larger spatial or temporal scale (Egger and Hibbett 2004).

Responsiveness to AM symbiosis is influenced by plant and fungal factors. Plant factors that influence the response to AMF colonization are for example plasticity of root to shoot ratio, length and branching of the root system as well as length and density of the root hairs (Jakobsen et al. 2002, Smith and Smith 2011). On the other hand, the identity (species and genotype) of the fungal partner also influences the mycorrhizal responsiveness (Munkvold et al. 2004). Important physiological characteristics of AMF are growth rates and densities of the extraradical mycelium (Jakobsen et al. 2002). However, the interaction of both partners in sense of development of the symbiotic interface and rate of resource exchange determines the compatibility of the association, which can greatly vary between different combinations (Smith et al. 2004, Smith and Smith 2011). Finally, the outcome of the symbiosis is largely dependent on environmental conditions, such as availability of different nutrients (Johnson et al. 1997). More precisely, a trade balance model based on the relative availability of P, N and C elucidates the interplay of the different resources in the AM symbiosis (Johnson 2010). Strong mutualistic interactions are predicted when only P is limited due to the fact that N deficiency restricts plants photosynthetic C allocation. In contrast, parasitism is

predicted when neither P nor N are limited and the plants gain no benefit from C allocation to the fungus.

Whether a plant is profiting from the AM symbiosis or not, depends on the net benefit, the relation between investment and beneficial return. Indeed, the C used by the fungus represents a considerable cost to the plant, which may or may not be compensated by the benefit in return. Net benefit is usually determined in terms of mycorrhizal growth response and interpreted as potential fitness gain for the plant (Koide and Elliott 1989). However, in natural systems, plant fitness is typically measured by survival and fecundity, whilst only in agricultural systems biomass seems to be an appropriate parameter (Johnson et al. 1997). Costs are traditionally expressed in terms of photosynthetically fixed C allocated to the fungus. But AMF also influence plant morphology in particular the root to shoot ratio. Thus, if formation of AM symbiosis results in a reduction of root growth compared to an equivalent non-mycorrhizal plant, the actual costs to the plant are less than the loss of C to the fungus (Johnson et al. 1997). Moreover, the C use efficiency of mycorrhizal plants can be higher than of non-mycorrhizal plants (Tinker et al. 1994). The higher efficiency may be caused by the fact that foraging the soil by hyphae involves smaller C costs per absorbing area than by roots.

AM benefits are usually determined as mycorrhizal responsiveness of a plant, which arises largely from increased uptake of limited nutrients *via* AMF hyphae (Smith and Read 2008). Nevertheless, tracer studies have shown that mycorrhizal nutrient acquisition can be hidden (Smith et al. 2003b, 2004). This means, that even in case of no additional nutrient supply, AMF can provide most of the nutrients to the plants and therefore provide beneficial services. Furthermore, AMF enhance besides plant nutrition also the resistance against drought and soil pathogens (Auge 2001, Graham 2001). Generally speaking, the costs and benefits of the AM symbiosis are rather diffuse, and therefore it is difficult to differentiate between antagonistic and mutualistic associations.

Specificity of plant-fungal interaction

The establishment of AM is not only dependent on the capability of both protagonists to form the symbiosis, but also on the specificity and compatibility of the interaction. The aspect of specificity considers the range of potential partners that are compatible for plant and fungal symbionts and indicates whether a given species of fungus can form an AM association with more than one plant species or whether a given plant species associates with more than one species of fungus.

Experiments under controlled conditions examining single plant-fungus interactions have shown that there is no absolute specificity in the AM symbiosis, because almost any plant species that is capable of forming the AM symbiosis can be colonized by almost any AMF strain (Smith and Read 2008). However, this simple demonstration that has been made many times, but only indicates that under such artificial conditions no specificity is to expect (Sanders 2002). Further evidence for the lack of specificity is given by the systematics of the potential plant and fungal partners, since AMF comprise a relatively small number of genera and species compared to the vast diversity of plants (Law 1985). Besides experiments under controlled conditions also molecular investigations on field sites revealed that a single plant can be colonized by up to 20 different AMF species (Fitter 2005). It has been assumed that AMF, like many soil-inhabiting fungi, have relatively ineffective dispersal mechanisms and thus, benefit from low specificity by enabling them to access a wide range of plant species (Smith and Read 2008). Furthermore, mutualistic symbioses are generally not expected to show the same specificity as pathogenic symbioses (Law and Lewis 1983, Douglas 1998).

However, for many plant species the engagement in AM symbiosis clearly leads to fitness advantages, which are highly dependent on the identity of the fungal partner (Carey et al. 1992, Streitwolf-Engel et al. 1997, van der Heijden et al. 1998a). Thus, selective pressure should drive plants to from specific associations with more beneficial AMF (Sanders 2002). Indeed, it is becoming increasingly apparent that in field distinct AMF communities are present in the rhizosphere of different plants (Bever et al. 1996, Montesinos-Navarro et al. 2012), and that there is a certain specificity in the association to plant species (Helgason et al. 2002, Davison et al. 2011). Such preferences for individual AMF have also been reported under controlled conditions reflected in highly different degrees of root colonization (Smith et al. 2009).

1.5 ROLE AND FUNCTION OF AMF IN PLANT COMMUNITIES

Impact on plant coexistence

Populations of plants and AMF live together in communities and compete for limiting resources. According to the resource competition theory of Tilman (1982), the presence of AMF affects plant coexistence by enabling mycorrhizal plants to become more competitive for belowground resources (Allen and Allen 1990a, van der Heijden 2002). Simultaneously, different AMF compete among each other for C within roots and for mineral nutrients in the rhizosphere (Maherali and Klironomos 2007). Interestingly, trade-offs between the benefits AMF provide for plants and their competitive ability in the roots can occur. A study comparing three different AMF species has shown that the fungi compete for root space, and that the best competitor was the worst mutualist (Bennett and Bever 2009). In addition, the worst competitor within the root was the best mutualist. However, the interactions between plants and AMF populations may stabilize or destabilize the whole community (van der Heijden et al. 1998a, Vogelsang et al. 2006, Bever et al. 2010, Wagg et al. 2011). Bever (1999) developed a general model that identifies potential stabilization or destabilization effects of mutualism, which influence the diversity of a plant community. The model is based on the observation that plant and AMF populations are interdependent and it distinguishes between two dynamical outcomes. A relatively reciprocal delivery of benefits between plant and fungi promotes the more beneficial symbionts and results in a positive feedback dynamic (Bever et al. 1997), whereas negative feedback occurs if the delivery of benefit in the AM symbiosis is highly asymmetric and less beneficial symbionts are favored (Bever 2002, Klironomos 2002). These two processes have very different effects; positive feedback stabilizes the established community, but reduces diversity of the system. Negative feedback generates oscillations in local abundances of plant and AMF and so promotes the diversity in the community (Bever 1999).

The diversity of the AMF population has substantial impact on diversity and productivity of plant communities (van der Heijden et al. 1998b, Wagg et al. 2011). The observed productivity increase of the whole plant community indicates facilitative effects mediated by the AM symbiosis. Facilitation is defined, in contrast to competition, as positive interactions between plants that are mediated through changes in the abiotic environment or through other organisms (Brooker et al. 2008). However, a

generalization that AMF promote plant diversity and productivity is not possible, because the impact of AMF on plant community structure is contradictory and repeated patterns do not really occur. On the one hand, it has been shown in field and greenhouse experiments that presence of AMF enhances plant diversity in European grasslands (Grime et al. 1987, van der Heijden et al. 1998b). In contrast, AMF reduced the plant diversity in an experiment conducted in the American tall grass prairie (Hartnett and Wilson 1999). Indeed, when competing plants differ in their mycorrhizal responsiveness, the diversity will be promoted by AMF when the inferior competitor is more responsive, but plant diversity will be decreased when the superior plant is more responsive (Hart et al. 2003). Furthermore, the studies mentioned above indicate that the relative dominance of a plant in a community does not determine how much benefit it receives. In contrast, the identity of the dominant plant and its mycorrhizal responsiveness appears to determine the impact of AMF on plant diversity (van der Heijden and Horton 2009). Further experiments comparing the effect of AMF on plant pairs have shown that the mycorrhizal responsiveness is also highly depending on neighboring plant species (Marler et al. 1999, Scheublin et al. 2007). Hence, it is not possible to predict the influence of AMF on plant competition in a community on the basis of mycorrhizal growth responses of individual plants (Facelli et al. 2010).

Common mycorrhizal networks

Although plant-fungal interactions exhibit a certain degree of specificity, in a plant community several plant individuals also from different species can be colonized by the same fungus and thereby be interconnected by a so-called common mycorrhizal network (CMN; Figure 1.4). Direct physical or functional evidence of interplant linkages by CMNs has been reported in studies using microscopic and autoradiographic tracings within transparent microcosms (Francis and Read 1984, Newman et al. 1994). CMNs arise not only from single AMF colonizing neighboring roots, but also from anastomoses connecting previously separated but genetically close mycelia (Giovannetti et al. 2004). These interplant linkages exhibit high functionality and allow neighboring plants to use the entire CMN for their nutrient acquisition (Mikkelsen et al. 2008). Remarkably, the interconnectedness of CMNs is dependent on the identity of the involved fungal partners (Avio et al. 2006). Moreover, architecture of mycorrhizal networks varies also at higher

taxonomical resolution. Networks of the Glomeraceae are thought to be better interconnected compared to those of the Gigasporaceae, because they form more anastomoses (de la Providencia et al. 2005, Voets et al. 2006). The interconnectedness of neighboring plants helps to understand the effect of neighboring plants on mycorrhizal responsiveness. In an experiment with a plant pair comprised of a forb and a grass, the presence of a CMN increased the growth of the forb by facilitating its P uptake (Zabinski et al. 2002). The P access of the forb was increased over a wider area of soil including the neighbor's rooting zone, resulting in enhanced P acquisition.

CMNs can have several effects on plant communities; e.g. assistance in seedling establishment by allowing seedlings to become connected to an established CMN, reducing nutrient loss of the ecosystem by keeping the nutrients in the living biomass, increasing productivity and stability of ecosystems and promote plant interaction by allowing resource exchange directly from plant to plant (Simard et al. 2002). Improvement in seedling establishment has only been shown in some cases (van der Heijden 2004), but not in others (Kytoviita et al. 2003). Thus, two studies proposed seedling support by CMN to be less likely due to a preferential allocation of resources to the larger plant (Nakano-Hylander and Olsson 2007, Pietikainen and Kytoviita 2007).

The best-studied potential effect of CMNs is the interplant resource transfer (Figure 1.4B). Besides the transfer of P and N (Johansen and Jensen 1996, Wilson et al. 2006), the transfer of C awoke the interest of the scientific community. First evidence for interplant C transfer *via* CMN was provided by Simard et al. (1997) in a system with ectomycorrhizal fungi. They could show by isotopic labeling that the C transfer between the two tested plants was bidirectional, but one plant experienced a net C gain of up to 10 %. Interplant C transfer has also been shown in several studies with AMF, where the C flow was tracked either by labeling with the radioactive isotope ^{14}C (Francis and Read 1984, Lerat et al. 2002), or by using the natural abundance of the stable isotope ^{13}C differing between C_3 and C_4 plants (Watkins et al. 1996, Fitter et al. 1998, Carey et al. 2004). However, direct C transfer has never been shown in ecological relevant amounts indicated by the marginal benefit of the transferred C on the growth of the receiver plant (Robinson and Fitter 1999). One of the reasons for the missing effect on plant growth has been shown in a more mycocentric approach where it was shown that the transferred C remained in the fungal tissue (Pfeffer et al. 2004, Voets et al. 2008, Lekberg et al. 2010).

However, even when transferred C remains in the fungal tissue and is not transferred into the receiver plant, the plant may get a benefit, because the C demand of the fungal partner is subsidized by the donor plant (Simard and Durall 2004). Such unequal C allocation for the growth and maintenance of a CMN could greatly influence the coexistence of plants sharing a mycorrhizal network (Jakobsen 2004, Selosse et al. 2006). Thus, a plant providing relatively little carbon to the CMN would obtain its mineral nutrients at a lower expense than other interlinked plants and thereby achieve competitive advantage. Moreover, plant growth in many ecosystems is not light but nutrient limited. Hence, AM symbiosis may exist without causing significant costs to either partner when photosynthesis is able to cope with additional C costs caused by the fungus (Smith et al. 2009).

CMNs also have an impact on the ecology of the fungi. Reduced C allocation of the plant, induced by shading or leaf clipping, results usually in a reduction of colonization level of AMF in single plant-fungus interaction (Tester et al. 1985, Heinemeyer et al. 2004). In contrast, C limitation in a CMN did not affect the colonization level in the root of the C-limited plant. Thus, the fungus received still enough C of the interconnected plants (Lekberg et al. 2010). They propose that in a mycocentric view of CMNs, C-delivering plants are active symbiotic partners exchanging C for nutrients, while C-limited plants are storage units. Storage of C within plant roots is probably safer than in the rhizosphere where a lot of potential consumers live (Bakhtiar et al. 2001).

Mycoheterotrophy

A fascinating group of plants has lost their chlorophyll and hence the ability for autotrophic C allocation and making them heterotrophic organisms. These achlorophyllous plants can be separated into two categories, parasitic and mycoheterotrophic (MH) plants. The existence of the parasitic plants is sustained by direct physical attachment to autotrophic plants, which enables the transfer of organic C through haustoria-like structures (Sitte et al. 1998).

In the other category, heterotrophy is sustained by forming associations with fungi (Leake 1994). More than 400 achlorophyllous plant species are MH. The majority of MH plants are restricted to the tropics, nevertheless, MH Ericaceae and some Orchidaceae occur also in temperate forests (Merckx et al. 2009). In over 90% of the cases the ultimate C source of MH plants is generally unknown (Leake 2004). Historically, it was assumed that MH plants obtain their C directly from soil organic matter and were therefore described as “saprophytes” (Leake 1994).

However, early investigators demonstrated the presence of fungal filaments closely associated with the root systems. Moreover, by using molecular markers it was becoming clear that most of MH plants are receiving C and nutrients from mycorrhizal fungi (Merckx et al. 2009) and are therefore thought to be indirectly epiparasitic to autotrophic plants (Figure 1.4C; Björkman 1960). As an alternative to associations with mycorrhizal fungi, some MH orchids are specialized on litter and wood-decay fungi (Leake 1994, Ogura-Tsujita et al. 2009).

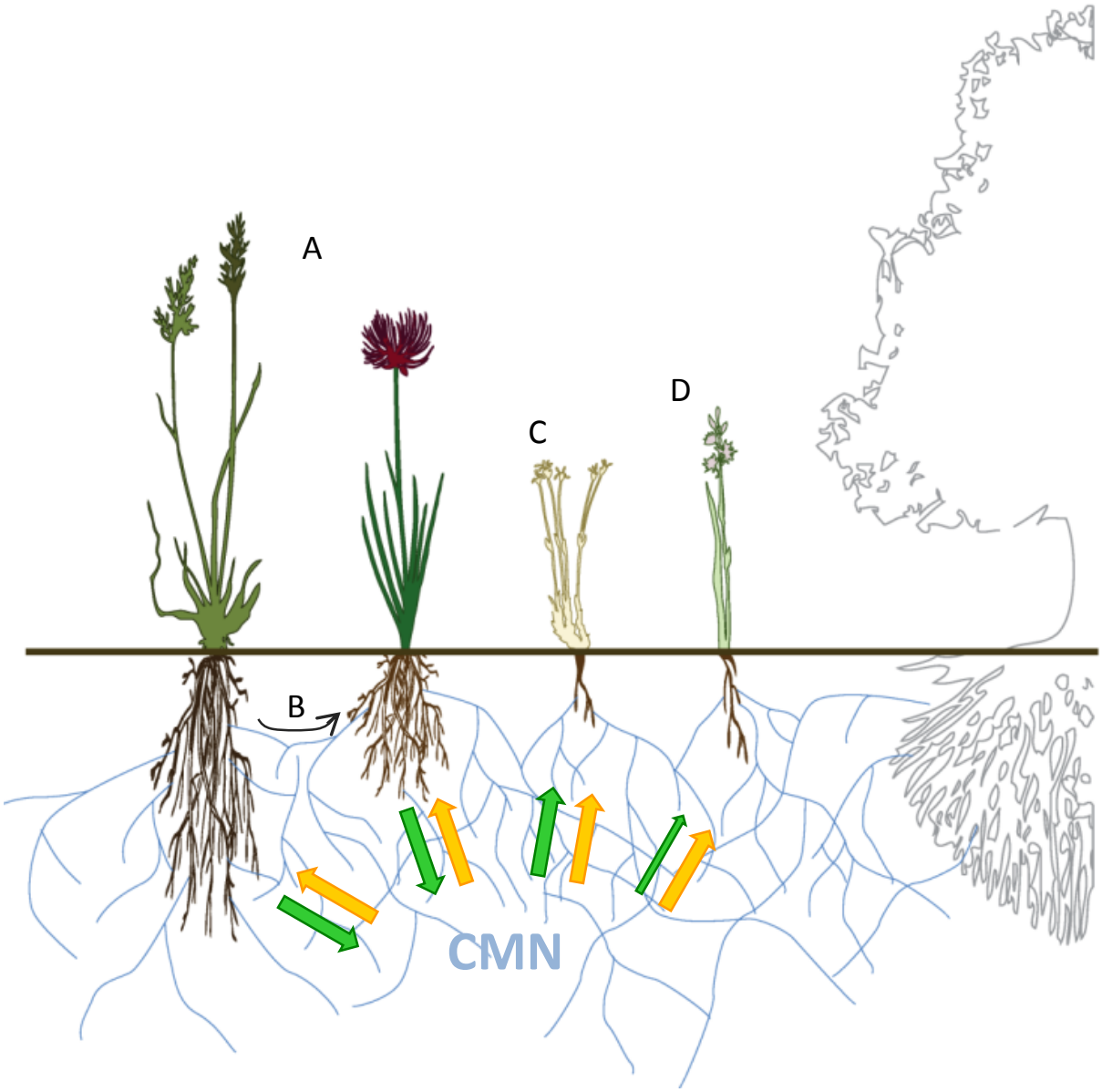

Figure 1.4. One mycorrhizal fungal individual colonizes different plant species and forms a common mycorrhizal network. (A) Autotrophic plants invest carbon (green arrows) to the CMN, and receive in return soil nutrients (yellow arrows). (B) Further-more, hyphal connections are believed to facilitate interplant transfer of carbon and nutrients. (C) Achlorophyllous mycoheterotrophic plants are not able to fix carbon by photo-synthesis and thus using the CMN as nutrient and exclusive carbon source. (D) Mixotrophic plants are only weakly photo-synthetically active and exploiting the CMN besides for nutrients also partially for carbon.

MH plants are cheating the mycorrhizal associations and may make use of the diffuse relations in CMNs. Moreover, they represent the unequivocal evidence for the significance of interplant C transfer *via* CMNs (Smith et al. 2009). Nevertheless, the C transfer from the fungus to the plant is still obscure, but it is assumed that the plant actively digests the fungus (Imhof 1999). The physiology of MH plants remained nearly unexplained until the recent application of stable isotopes. The analysis of the natural abundance of ^{13}C and ^{15}N has been extensively used to provide insights into C and N sources of MH plants in association with ectomycorrhizal fungi (e.g. (Gebauer and Meyer 2003, Roy et al. 2009). More recent studies showed that also green orchids phylogenetically related to MH species are able to exploit C from a CMN shared with surrounding trees (Julou et al. 2005). Partially autotrophic plants, which use CMN-derived C as part of their C resources, are classified as mixotrophic plants (Figure 1.4D; Selosse and Roy 2009).

MH plant species associated to AMF have been found in the dicotyledonous families Polygalaceae and Gentianaceae, and in the monocotyledonous families Triuridaceae, Petrosaviaceae, Corsiaceae and Burmanniaceae (Leake 2004). The presence of AMF in the roots of those plants was confirmed by structural analysis (Imhof 1999, Imhof and Weber 2000, Franke 2002) and with the help of molecular markers (Bidartondo et al. 2002, Franke et al. 2006). Only recently, the first physiological evidence for MH plants associated to AMF was given by comparison of the natural abundance of ^{13}C and ^{15}N (Merckx et al. 2010). Interestingly, MH plants appear to be specialized on an extremely narrow lineage of AMF species. Molecular analysis of the gentianaceous genera *Voyria* and *Voyriella* showed that they are associated with closely related *Glomus* species (Bidartondo et al. 2002). In another study, again, four MH plants of the genus *Afrothismia* (Brumanniaceae) were colonized by distinct *Glomus* species (Franke et al. 2006). Furthermore, it has been shown that MH and a green *Burmannia* species were associated with distinct AMF species (Merckx et al. 2010). Thus, in contrast to the AM symbiosis found for autotrophic plants, specificity in associations of MH to AMF appears to be rather high.

1.6 PHOSPHORUS ACQUISITION BY PLANTS

Phosphorus in the rhizosphere

Phosphorus (P), one of plant's essential macronutrients, is an integral part of energy metabolism of plants, a constituent of nucleic acid and of membranes. Moreover, P is one of the most critical growth limiting factors in a variety of natural and agro-ecosystems (Marschner 1995). The primary source of P for plants is inorganic phosphate (P_i). Thus, effective acquisition of P_i from the soil is crucial for the plant development. However, P is reasonably abundant in soil, where it primarily occurs as P_i in minerals derived from bedrock (Marschner 1995). But, the abundance of P_i is decreasing with soil age due to leaching in the course of weathering and soil formation (Lambers et al. 2008). Furthermore, organic phosphate (P_o) gradually accumulates on the expense of P_i as a consequence of consumption of P_i by various organisms colonizing the soil (Richardson et al. 2004). Hence, at later stages of development soils become P limited, while at early stages of development soils are primarily N limited (Vitousek and Farrington 1997, Lambers et al. 2008).

P_i is held very firmly in largely insoluble forms, such as Calcium, Iron and Aluminum phosphates and is often chemically bound to the surface of clay minerals (Marschner 1995). Moreover, P_i exchanges very slowly with the soil solution leading to a soil solution concentration between 5 and 10 μM (Schachtman et al. 1998). As a consequence, P_i may be present in relatively large amounts in the soil, but much of it is tightly bound and unavailable for plants. The availability of P_i can be affected by many abiotic factors. For example, an increase in soil pH can drastically rise P_i availability due to an enhanced desorption of P_i by changes in surface charge of clay minerals (Hinsinger 2001). Additionally, root exudates, such as carboxylates, can compete with P_i for the adsorption on a charged surface. The so induced ligand exchange promotes P_i desorption and enhances thereby P availability.

The predominant forms of P_o in the soil are phytates, phospholipids and nucleic acids. P_o does not seem to be a major pool directly available to plants, although some plants have the capability to excrete acid phophatases hydrolyzing P_o (Vance et al. 2003). However, for most plants the access to the P from P_o pools is dependent on hydrolysis by microorganisms. A wide range of microorganisms is able to solubilize P_i from organic

material including bacteria (e.g. *Actinomycetes, Pseudomonas,* and *Bacillus* spp.) and fungi (e.g. *Aspergillus* and *Penicillium* spp.; Richardson and Simpson 2011). Also AMF seem to increase the capability of plants to access P from P_o, but it is difficult to separate direct enzymatic effects from indirect effects such as increased microbial activity promoted by AMF (Joner and Jakobsen 1994, Joner and Johansen 2000, Koide and Kabir 2000).

Soluble P_i in the soil solution is present as free orthophosphate. Since orthophosphate is rapidly fixed by adsorption on soil surface, precipitation of mineral phosphates and usage by soil organisms, it is virtually immobile in soil (Hinsinger 2001). Therefore, it is apparent that mass flow of soil solution is unable to supply plant roots with a sufficient amount of P_i. This high immobility of orthophosphate in combination with a rapid absorption results in the development of a depletion zone around the roots (Figure 1.5; Marschner 1995). As a consequence, the P pool available for plants is largely dependent on the extent of root system and roots have to proliferate to increase the pool of available P (Hinsinger et al. 2011).

Plant phosphorus uptake

During P_i uptake from the soil solution, plants have to deal with several challenges (Smith et al. 2003a). Firstly, P_i has to be taken up against a huge concentration gradient; the P_i concentration in plant cells is 1000-fold higher than in the soil solution. Additionally, orthophosphate is negatively charged and has to be transported across the plasma membrane exhibiting an inside-negative electric potential. Thus, P_i uptake across the plasma membrane into the plant cell will not occur passively. It requires a high affinity and energy driven transport process, which is mediated by two collaborating membrane-spanning proteins, a phosphate transporter and an H^+-ATPase (Smith et al. 2003a, Bucher 2007). The H^+-ATPase pump is used to generate the electrochemical gradient across the plasma membrane at the expense of ATP. The developing proton gradient is the driving force for the P_i influx via the H^+/P_i symporter encoded by genes belonging to the *Pht1* family (Karandashov and Bucher 2005).

P_i uptake in plant roots begins by apoplastic transport through the intercellular space between the walls of root epidermal and cortical cells (Smith et al. 2003a). Soil solution is able to move towards the center of the roots through the apoplastic way until it meets the Casparian band situated at the walls of endodermis cells that surround the central

stele. The boundary between apoplasm and symplasm in roots is the plasma membrane of root epidermal and cortical cells. Once inside the symplasm, P_i can move through plasmodesmata without encountering further membrane barriers (Clarkson 1993). Thus, the transfer across the plasma membrane of the root epidermal, cortical and endodermis cells is a crucial step in P_i uptake and is mediated by specialized members of the *Pht1* gene family (Bucher 2007). In the symplasm, P_i is transferred from cell to cell towards the endodermis, where it gets translocated into the xylem of the central stele. The xylem transports P_i with water and other mineral nutrients, and distributes it throughout the plant (Sitte et al. 1998).

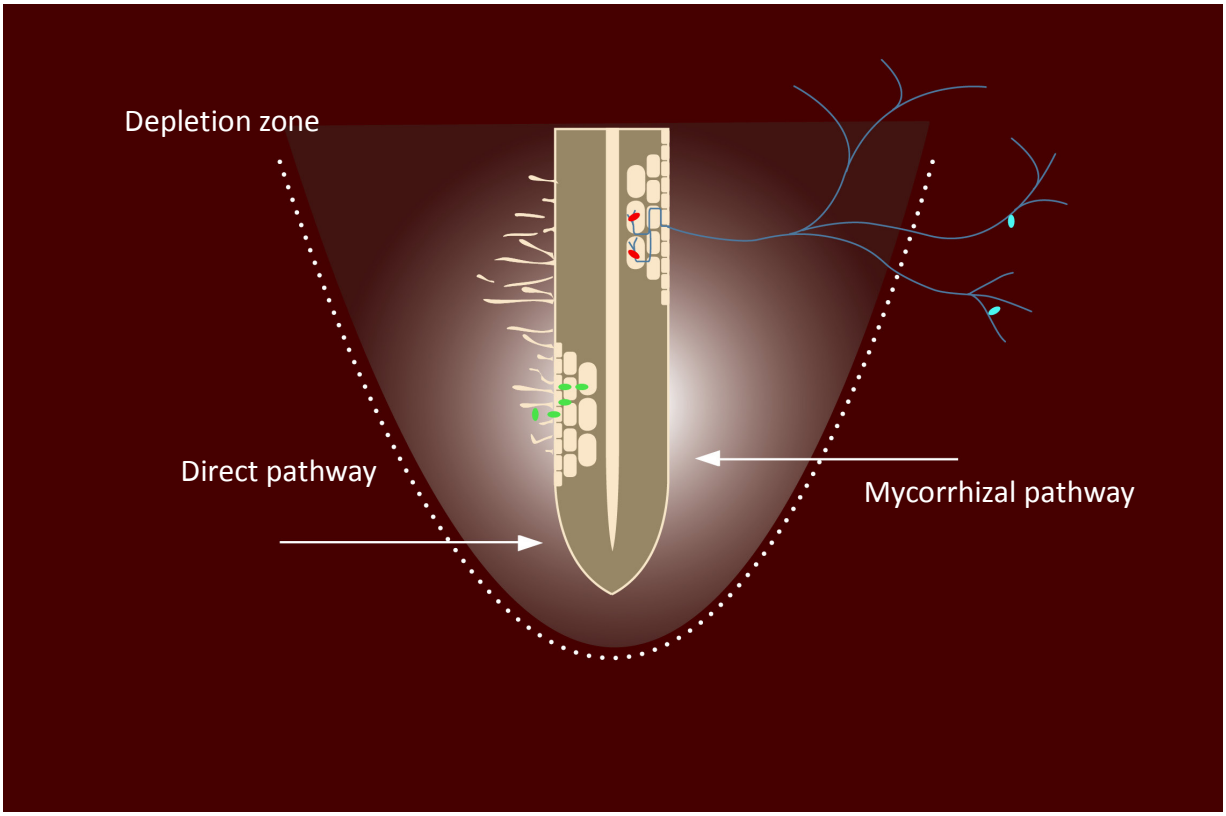

Figure 1.5 The two P uptake pathway of mycorrhizal plants. In the direct pathway, P_i is absorbed from the rhizosphere by plant P_i transporters in epidermis and root hairs (green circles) close to the root surface. Uptake is normally faster than replacement by diffusion from the bulk soil, resulting in reduced P_i concentrations (depletion) close to the roots. In the mycorrhizal pathway, P_i is taken up into AMF hyphae by fungal P_i transporters (blue circles) beyond roots depletion zone and translocated to intracellular fungal structures (arbuscules and hyphal coils) in root cortical cells. Plant P_i transporters (red circles), induced in colonized cells, transfer P_i from the interfacial apoplast to plant cortical cells. Modified after Smith and Read (2008).

Mycorrhizal phosphorus uptake

At the soil root interface, mycorrhizal plants can absorb P_i through two different pathways; (i) directly *via* the epidermis including root hairs, (ii) or *via* the mycorrhizal pathway starting at the hyphae of the extraradical mycelium (Figure 1.5). Although both pathways absorb the same form of P_i, in association with AMF, plants dramatically increase their P supply (Marschner and Dell 1994). Fungal hyphae are foraging the soil for nutrients beyond the depletion zone of the roots and having much smaller diameters than roots, they get access to narrower soil pores, which increases the explored soil volume (Figure 1.5; Drew et al. 2003, Schnepf et al. 2011).The absorbed P_i is translocated within the AMF hyphae towards the roots. This process is much faster than diffusion through the soil (Smith and Read 2008). Consequently, hyphal transfer can overcome the limited P availability for plants resulting from the fast development of a depletion zone around roots.

P_i is probably absorbed as orthophosphate and is mediated by P_i transporters closely related to the *Pht1* family of plants (Figure 1.6). All fungal P_i transporters are strongly expressed in extraradical hyphae and appear to be high affinity transporters, ready for taking up P_i from low concentrations in the soil solution (Harrison and Vanbuuren 1995, Maldonado-Mendoza et al. 2001, Benedetto et al. 2005). In the fungus, orthophosphate is transferred to vacuoles, where it gets converted to polyphosphate (poly-P; Ezawa et al. 2004). Poly-P is moving within vacuolar compartments from extra- to intraradical mycelium. There it gets hydrolyzed and exported as P_i across the periarbuscular membrane by AM-specific P_i transporters (Figure 1.2; Javot et al. 2007b, Smith et al. 2011). It is generally accepted that P transfer from fungus to plant occurs at arbuscules. The presence of AM-inducible P_i transporters and H^+-ATPases on the periarbuscular membrane confirms this assumption. However, the evidence that would exclude intercellular hyphae from P_i transfer is still lacking (Smith and Read 2008).

Phosphate transporters in plants

Plants hold several P_i transporters distributed throughout the plant exhibiting distinctive functions and kinetic properties dedicated for different P_i concentrations (Smith et al. 2003a). The use of P_i within the plant requires transport through membranes wherever symplastic connections do not occur. Thus, membrane transport systems are not only required for P_i uptake, but also for the distribution and remobilization of P_i throughout plant, as for example in case of in- and efflux of xylem and phloem. Additionally, phosphate transporters are crucial for intracellular movement of P_i between different compartments; such as transport between the vacuole and cytosol across the tonoplast, transport between the cytosol and the stroma of the chloroplasts, and transport of P_i between cytosol and mitochondria. The plant P_i transporters are classified in three families: Pht1, Pht2 and Pht3 (Figure 1.6; Rausch and Bucher 2002, Smith et al. 2003a). The most prominent P_i transporter group of plants is the Pht1 family, which is described in detail at a later point. Members of the Pht2 family are located in the chloroplast (Ferro et al. 2002, Versaw and Harrison 2002) and functional analysis indicated that they are low-affinity H^+/P_i symporters (Daram et al. 1999). The Pht3 family harbors P_i transporters highly conserved within the mitochondrial transporter family (Takabatake et al. 1999). However, the function of these mitochondrial transporters yet remains unknown.

Pht1 transporters are secondary transporters belonging to the H^+/P_i symporter family within the major facilitator superfamily. The major facilitator superfamily consists of several distinct families each transporting a single class of compounds. It is believed that all the members of this superfamily arise from divergence after tandem duplication events of a primordial protein (Pao et al. 1998). The Pht1 transporters share the structural similarity of 12 transmembrane (TM) domains with the other members of the major facilitator superfamily. However, the Pht1 protein topology has been demonstrated only *in silico*, while experimental evidence is still lacking (Bucher 2007). Multiple alignments of Pht1 transporters have revealed the presence of a highly conserved amino acid sequence (GGDYPLSATIxSE) situated in the fourth putative TM domain (Karandashov and Bucher 2005).

Plants have several *Pht1* genes, but the number of genes differs between species. For instance, the completely sequenced genomes of rice (*Oryza sativa*) and Arabidosbis revealed that rice exhibit 13 *Pht1* genes (Paszkowski et al. 2002), whilst only nine have been found in Arabidobsis (Rausch and Bucher 2002). Most *Pht1* genes are strongly expressed in the epidermal cells, including roots hairs, and the cortex cells of the root indicating a role in direct P_i uptake (Daram et al. 1998, Liu et al. 1998a, Chiou et al. 2001, Ai et al. 2009). In agreement, analysis of loss-of-function and knockdown mutants of Arabidopsis and rice clearly demonstrated that Pht1 transporters are responsible for a substantial portion of total P uptake (Shin et al. 2004, Ai et al. 2009). However, expression of Pht1 transporters has also been observed in leaves and inflorescences. This indicates roles of *Pht1* genes besides P_i uptake of soil solution as for example remobilization of P_i from leaves (Rae et al. 2003) or P_i uptake in the elongation tube of pollen (Mudge et al. 2002, Nagy et al. 2006). Other *Pht1* genes appear to be more generally expressed all over the plant indicating an involvement in P homeostasis (Jia et al. 2011). This batch of P_i transporters also displays a diverse functionality regarding the affinity for P_i. Many plant P_i transporters have been functionally characterized by heterologous expression in yeast (*Saccharomyces cerevisiae*) or by overexpression in tobacco suspension-cultured cells. The P_i affinity is ranging from 3 to ~700 μM, indicating the presence of low- and high-affinity P_i transporters within the Pht1 family (Mitsukawa et al. 1997, Harrison et al. 2002, Rae et al. 2003, Nagy et al. 2005, Liu et al. 2008, Wu et al. 2011).

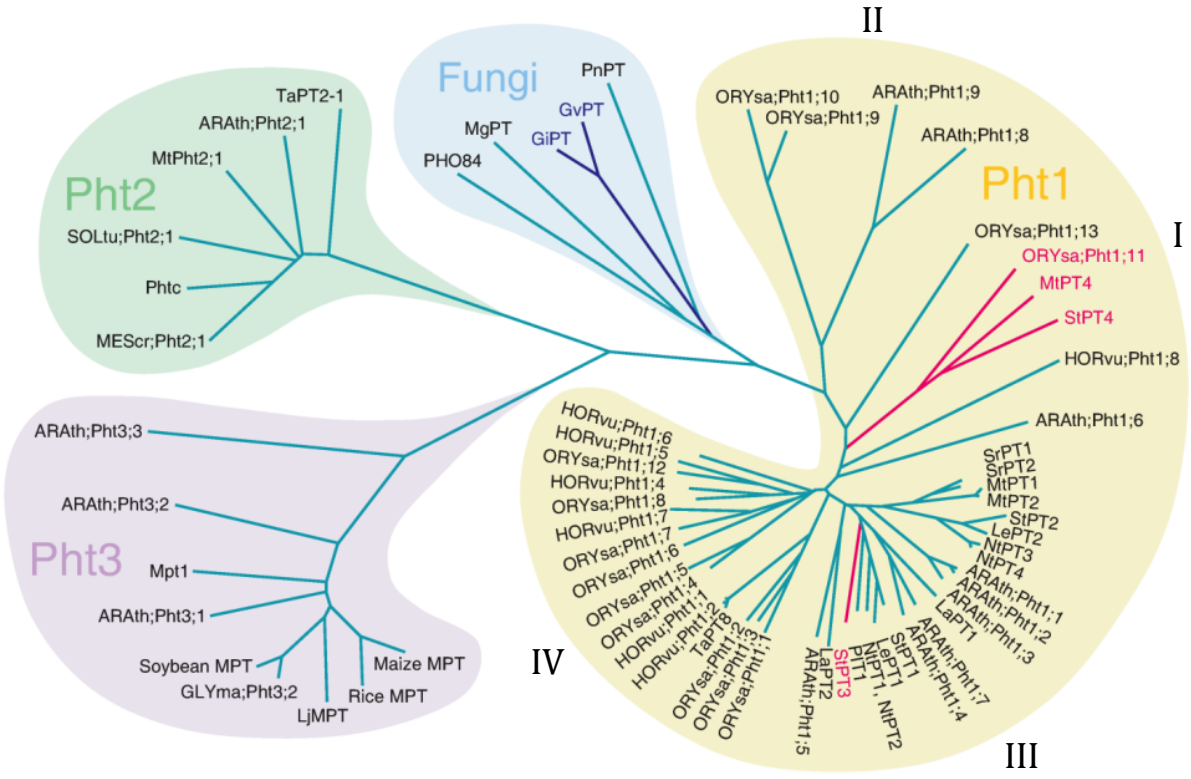

Figure 1.6 Unrooted phylogenetic tree of plant and several fungal phosphate transporters. Plant phosphate transporters are grouped into three families, Pht1, Pht2 and Pht3, each containing a group of proteins likely to have similar functions that are distinct between the families. Pht1 and Pht2 proteins have been localized to the plasma membrane and the inner envelope of chloroplasts, respectively, while Pht3 proteins are mitochondrial phosphate transporters. AM-inducible plant phosphate transporters are shown in red. AM-fungal phosphate transporters are shown in blue. Roman numbers indicate four different plant Pht1 subfamilies. Modified after Karandashov and Bucher (2005).

AM-inducible phosphate transporters

In mycorrhizal plants, there exists a small group of *Pht1* genes, which is induced by the presence of the AM symbiosis. On the one hand, there is an AM-specific group expressed strictly in response to AM symbiosis. Such AM-specific *Pht1* genes have been identified first in *Medicago truncatula* (*MtPT4*; Harrison et al. 2002) and rice (*OsPT11*; Paszkowski et al. 2002), followed by many others (Appendix A1, Table A1.1). On the other hand, there are several *Pht1* genes, which are up-regulated in response to formation of AM symbiosis, but have a basal expression in non-mycorrhizal roots (Rausch et al. 2001, Guimil et al. 2005, Maeda et al. 2006).

Intriguingly, a large majority of AM-inducible Pht1 transporters clusters into the distinctive subfamily I containing strictly proteins of plant species that are capable to form AM symbiosis (Figure 1.6). This subfamily harbors AM-inducible transports of monocots and dicots and likely will be found in the majority of all potential AM plants (Karandashov and Bucher 2005, Yang and Paszkowski 2011). The high amino acid sequence similarity suggests that these AM-inducible transporters arose from the same primordial protein (Smith and Read 2008). By contrast, the AM-induced Pht1 transporters StPT3, LePT3 and LjPT3 group in subfamily III, which is evolutionary younger than the subfamily I indicated by harboring solely proteins from dicotyledonous species (Bucher 2007). This is an evidence for a separate evolutionary gain of AM-inducibility of genes involved in P_i uptake exhibiting more general expression patterns (Karandashov et al. 2004).

The heterologous expression of AM-inducible Pht1 transporters in yeast gives an unsteady picture; the AM-specific protein of *M. truncatula*, MtPT4, revealed low-affinity to P_i (Harrison et al. 2002), whereas the AM-up-regulated StPT3 of potato showed a ten times higher affinity (Rausch et al. 2001). The functional relevance of these data has to be confirmed in other AM-inducible Pht1 transporters, and probably in comparable heterologous gene expression systems. For several of the AM-induced Pht1 transporters, *in situ* hybridization and promotor::GUS fusion studies have shown that the corresponding genes are predominately or exclusively expressed in cortical cells harboring arbuscules (Rausch et al. 2001, Harrison et al. 2002, Glassop et al. 2005, Nagy et al. 2005, Maeda et al. 2006). Furthermore, immunolocalization of MtPT4 protein revealed that the transporter was located solely in the periarbuscular membrane, more precisely, at the fine branches of developing or mature arbuscules (Figure 1.2; Harrison et al. 2002, Pumplin and Harrison 2009). This indicates that the periarbuscular membrane is composed of at least two distinct domains, an “arbuscule branch domain” that is specialized for the symbiotic P_i transfer and an “arbuscule trunk domain” that shares features with the plasma membrane.

AM-induced Pht1 transporters are essential for P_i uptake *via* the mycorrhizal pathway (Figure 1.5). This has been shown by down-regulation of *MtPT4*, which has lead to premature death of arbuscules, to a decreased colonization level and ultimately, to an incomplete development of the AM symbiosis (Javot et al. 2007a). The mutants showed

significantly lower total shoot P content probably caused by the impairment of the symbiotic pathway also indicated by the accumulation of poly-P in the arbuscules. A similar phenotype was found for the knockdown mutant of *LjPT3* in terms of both reduced arbuscule development and reduced AM-mediated P uptake by *L. japonicus (Maeda et al. 2006).* In contrast, knockout of *LePT4* gene did not inhibit arbuscule development and P_i uptake *via* the AM pathway in tomato, probably because of functional overlap with the other AM-inducible Pht1 transporter LePT3 (Nagy et al. 2005). Nevertheless, the crucial role of AM-induced Pht1 transporters in tomato has also been shown on a *reduced mycorrhizal colonization* mutant, where only those fungi were able to develop arbuscules, which induced the expression of *LePT3* and *LePT4* (Poulsen et al. 2005).

In addition to AM-induced P_i transporters, the H^+-ATPases generating the electrochemical gradient across the plasma membrane are also showing certain inducibility to AMF colonization. In tobacco plants colonized by AMF, H^+-ATPases showed arbuscule specific expression and distinct location in the periarbuscular membrane (Gianinazzi-Pearson et al. 2000, Krajinski et al. 2002).

Regulation of phosphate uptake

P acquisition and metabolism are highly regulated for economical usage of this often-limited nutrient, and to provide homeostasis. Plants acquire and store excess P_i in the vacuoles, which is crucial for P homeostasis of plant cells (Raghothama and Karthikeyan 2005). The cytosolic P_i levels are maintained at the expense of vacuolar P_i during short-term deficiency (Bieleski 1973, Mimura et al. 1996). There is evidence for ATP-dependent P_i transport system across the tonoplast similar to the Pht1 transporters across the plasma membrane (Sakano et al. 1995). P_i concentrations within the cytosol are maintained in a narrow range by regulation of influx and efflux processes (Mimura 1999).

Physiological measurements have shown that P starved plants rapidly increase their capacity in P_i uptake. Transcriptional regulation of P_i transporters appears to be important in response to P limitation. The expression of the *Pht1* genes is regulated by negative feedback to P_i availability, in terms of strong up-regulation when plants are P_i starved and decreased expression in times of sufficient P_i supply (Smith et al. 1997, Liu

et al. 1998a, Liu et al. 1998b). Remarkably, the overall nutritional status of the plant also influences the regulation of *Pht1* genes. It appears that in case of growth limitations by another nutrient, such as N, P_i transporters fail to respond to P starvation (Smith et al. 2003a).

Not only AM-inducible *Pht1* genes are affected by AM symbiosis. The up-regulation of AM-inducible P_i transporters is often accompanied by the down-regulation of other P_i transporters, in particular those thought to be involved in direct P_i uptake (Liu et al. 1998b, Rausch et al. 2001, Paszkowski et al. 2002; Appendix A1, Table A1.1). On the other hand, the AM pathway is down-regulated when direct uptake increases due increased P supply (Nagy et al. 2009). This interplay between Pht1 transporters may reflect the balance between direct and mycorrhizal pathway of P_i uptake. There is also physiological evidence indicating that in presence of AM symbiosis the P_i absorption by root hairs and epidermis is substantially reduced (Smith et al. 2003b). It is still not clear whether the down-regulation results indirectly from the AM-induced improvement of P_i acquisition or from direct down-regulation by the plant as a response to the symbiosis (Smith and Read 2008).

The diverse pattern of *Pht1* gene expression indicates that these genes must be specifically regulated according to different tissue types, variable P_i availability and presence and absence of AM symbiosis. A detailed analysis of the promoter region of *HvPT1* of barley revealed that distinct regulatory domains are responsible for tissue specific expression in root (Schunmann et al. 2004). However, much better characterized is the regulatory system involved in *Pht1* gene expression depending on P availability in non-mycorrhizal plant (Figure 1.7). The transcription factor PHR1 binds the cis-acting element P1BS found in the promoter of most *Pht1* genes and activates the expression of the transporter genes (Rubio et al. 2001, Nilsson et al. 2007). The micro RNA (miRNA) 399, also induced by PHR1, negatively regulates the enzyme PHO2 that suppresses the expression of *Pht1* genes (Bari et al. 2006, Lin et al. 2008). Hence, accumulation of mi399 in P starved plants leads to a de-repression of *Pht1* genes and thereby to an increase in P_i uptake *via* the direct pathway (Chiou and Lin 2011). Furthermore, the interplay of miRNA399 and PHO2 is fine-tuned by non-coding RNA as for example IPS1 (Shin et al. 2006, Franco-Zorrilla et al. 2007, Lin et al. 2009). Although the P starvation response appears to be strongly transcriptionally regulated, also posttranscriptional regulation

seems to be important. This has been shown by proteomic analysis of genes involved in P starvation response in Arabidopsis and rice, where the transcript abundance was not necessarily indicative for protein accumulation (Fukuda et al. 2007, Tran and Plaxton 2008).

Interestingly, the regulation of *Pht1* genes upon P starvation and AMF colonization has several regulatory elements in common (Figure 1.7). For instance, the combination of the regulatory element P1BS and a recently found AM-specific motif, MYCS, is required for activation of the AM-inducible *Pht1* genes in solanaceous species (Chen et al. 2011). The requirement of P1BS for the AM-inducibility of P_i transporters directly interlinks it with the P starvation response. This could explain the lack of AM-inducibility under high P_i supply, because PHR1 is suppressed (Nagy et al. 2009, Chen et al. 2011). However, there are additional mechanisms to sustain the AM symbiosis even in spite of a high P status. For instance, it has been shown that miRNA399 accumulates in shoots of AM plants, which may be translocated to the roots *via* the phloem (Branscheid et al. 2010). Furthermore, it has been shown that Mt4, a non-coding RNA homologous to IPS1 of *M. truncatula*, is rapidly down-regulated in the AM symbiosis (Burleigh and Harrison 1999). Both mechanisms keep the PHO2 activity low, which may be crucial to sustain the AM symbiosis also with a symbiotically increased P_i uptake. This is indicating that shared components between P starvation and AM signaling can also be differentially regulated according to a mycorrhizal or non-mycorrhizal state. Nevertheless, finding the regulatory elements responsible for the differentiation between P-starvation and AM-inducibility are needed to understand the interplay between direct and symbiotic P_i uptake pathway (Smith et al. 2011).

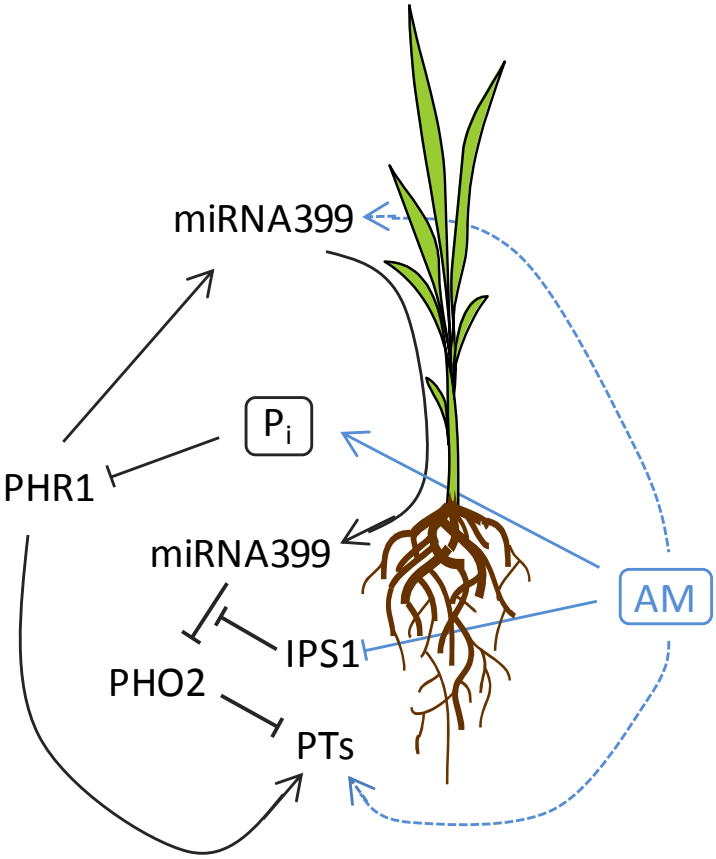

Figure 1.7 Overview of possible regulatory pathways of P star-vation and AM symbiosis. Low P increases the activity of the transcription factor PHR1, which binds to the P1BS element in the promoter region of inorganic phosphate Pht1 transporter genes (PT). PHR1 increases the expression of micro RNA (miRNA) 399. miRNA399 are probably largely synthesized in shoots, and induced by the formation of arbuscular mycorrhizal (AM) symbiosis. miRNA399 are tran-sported from shoot to root, where they reduce the activity of the enzyme PHO2 and hence increase the expression of PTs. Effects of RNA399 in reducing PHO2 activity can be quenched by non-coding RNA such as IPS1. However, AM symbiosis decrease IPS1 activity, probably to inhibit PHO2 activity also under high P_i supply. Modified after Branscheid et al. (2010).

1.7 AIMS OF THE THESIS

The major aim of this thesis arose from the fact that plants naturally share their AMF partner with their adjacent plants. Thus, plants are maintaining and utilizing a common fungal partner. The most important question posed thereby was: what are the terms of trade between plants and their shared fungal partner? Or more precisely, what is the C investment of a given plant into a CMN, and what is the nutritional benefit returned by the CMN? For this purpose, a model system comprised of sorghum (C_4 plant) and flax (C_3 plant) growing side by side in compartmented microcosms was established. This system allowed to assess the relative C investment of each plant by measuring the natural abundance of the C isotope ^{13}C in the CMN and to relate it to the CMN-mediated nutritional benefit by measuring uptake of ^{33}P and ^{15}N only accessible for AMF hyphae (Chapter 2).

A second focus of this thesis was to characterize the ability of sorghum and flax to acquire P_i in our model system. For this reason, P_i transporter genes belonging to the *Pht1* family were identified and characterized in both model plants. Finally, the expression of AM-induced *Pht1* genes was related to the functional P_i uptake obtained in the model system (Chapter 3).

MH plants receiving all their C and nutrients from the fungal partner represent an example of unidirectional resource exchange in CMNs. In order to shed light on the source of nutrition of MH plant species associated to AMF, the natural abundance of ^{13}C and ^{15}N was measured in the MH plants, in AMF spores and in the adjacent plants including herbaceous understory plants and trees (Chapter 4).

C flows from plants to AMF are of substantial amounts for the involved symbionts. However, the tracking of C flows in the AM symbiosis entails methodical difficulties. In a last part, we compare three different methods to assess the ^{13}C signature of C invested into the CMN, and we show that the analysis of the ^{13}C signature of the AMF biomarker fatty acid C16:1ω5 as performed in Chapter 2 is particularly suitable because it avoids much of the potential distortion of the ^{13}C signal by soil contaminants (Chapter 5).

2 MYCORRHIZAL NETWORKS: COMMON GOODS OF PLANTS SHARED UNDER UNEQUAL TERMS OF TRADE

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2.1 ABSTRACT

Plants commonly live in a symbiotic association with arbuscular mycorrhizal fungi (AMF). They invest photosynthetic products to feed their fungal partners which, in return, provide mineral nutrients foraged in the soil by their intricate hyphal networks. Intriguingly, AMF can link neighboring plants, forming common mycorrhizal networks (CMN). What are the terms of trade in such CMNs between plants and their shared fungal partners? To address this question, we set up microcosms containing a pair of test plants, interlinked by a CMN of *Glomus intraradices* or *G. mosseae*. The plants were flax (a C_3-plant) and sorghum (a C_4-plant), which display distinctly different $^{13}C/^{12}C$ isotope compositions. This allowed us to differentially assess the carbon investment of the two plants into the CMN through stable isotope tracing. In parallel, we determined the plants' "return of investment", i.e. the acquisition of nutrients *via* CMN, using ^{15}N and ^{33}P as tracers. Depending on the AMF species, we found a strong asymmetry in the terms of trade: flax invested little carbon but gained up to 94% of the nitrogen and phosphorus provided by the CMN that highly facilitated growth, while the neighboring sorghum invested massive amounts of carbon with little return but was barely affected in growth. Overall biomass production in the mixed culture surpassed the mean of the two monocultures. Thus, CMNs may contribute to inter-plant facilitation and the productivity boosts often found with intercropping compared to conventional monocropping.

2.2 INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) inhabit the soils of virtually all terrestrial ecosystems, forming symbiotic associations with most plants (Parniske 2008, Smith and Read 2008). The host plants incur substantial carbon costs to sustain this symbiosis (Jakobsen and Rosendahl 1990), but in return, they obtain multiple benefits from the fungal partners, above all, the provision of mineral nutrients. AMF may supply up to 90% of the host plant's nitrogen and phosphorus requirements (Smith and Read 2008). Moreover, AMF are important determinants of plant community structure and ecosystem productivity (Grime et al. 1987, van der Heijden et al. 1998b), and they represent a crucial asset for sustainable agriculture (Rooney et al. 2009). Typically, AMF exhibit little host-specificity; a single individual may form a common mycorrhizal network (CMN) between several co-existing plant individuals, even from different species (Whitfield 2007, Smith and Read 2008, Bever et al. 2010). Such CMNs may be enlarged through hyphal fusion of conspecific AMF (Giovannetti et al. 2004). The functionality of CMNs formed by fusion of two individual fungal networks by hyphal anastomoses, has been demonstrated by tracing nutrient allocation between individual host plants upon fusion of their associated CMNs (Mikkelsen et al. 2008).

The potential role and importance of CMNs is most apparent in the case of myco-heterotrophic plants. These plants connect themselves to an existing CMN to receive both carbon and mineral nutrients (Bidartondo et al. 2002, Courty et al. 2011). There is an ongoing debate whether carbon transfer through CMNs may also occur among autotrophic plants (Bever et al. 2010, Hodge et al. 2010). This is of a certain academic interest, but it may obscure a more general and obvious question arising from recent literature (Hodge et al. 2010, Hammer et al. 2011, Kiers et al. 2011, Smith and Smith 2011, Fellbaum et al. 2012). What are the terms of trade between plants and their shared fungal partners? Put another way, what is the "investment" of a given plant into a CMN (in the currency of assimilated carbon), and what is the "return of investment" in terms of mineral nutrients provided by the CMN? Indeed, different co-cultivated plants benefit differently from their CMN, depending on the AMF species involved, and these differences significantly affect plant co-existence (Zabinski et al. 2002, van der Heijden et al. 2003, Wagg et al. 2011). However, up to now, the relationship between carbon

investment and nutritional benefit of different plants engaged in a CMN has never been assessed.

To address the terms of trade in a CMN experimentally, we established a model system consisting of two plant individuals growing side by side in compartmented microcosms (Figure 1.1). The roots of the plants were confined to their respective "root hyphal compartments" (RHC). In experiments with AMF inoculation, however, the plants were able to connect through CMN in the "hyphal compartment" (HC) or in the "label-hyphal compartment" (LHC). We assessed the carbon investments of the single plants into the CMN through stable isotope tracing. We chose the C_3-plant flax and the C_4-plant sorghum for our experiments. Due to the different isotope fractionation during C_3 versus C_4 carbon fixation, these two species display distinctly different carbon isotope ratios ($\delta^{13}C$ ~33‰ for flax and ~14‰ for sorghum). This difference in ^{13}C signature of C_3 and C_4 plants has been widely used to track C flows in mycorrhizal symbioses (e.g. (Allen and Allen 1990b, Fitter et al. 1998). The plants were grown either in "monocultures", as pair of identical plant species, or in a "mixed culture", as pair of different plant species. We used two different AMF species in the experiments for inoculation, *Glomus intraradices* and *Glomus mosseae* (recently re-named *Glomus irregulare* (*Rhizophagus irregularis*) and *Funelliformis mosseae*, respectively (Schüssler and Walker 2010)). The chosen experimental setup allowed us to harvest the bulk of the CMN in the hyphal compartment (HC; Figure 2.1), and to estimate the respective carbon investment of the two plants into the CMN through the analysis of the $\delta^{13}C$ of isolated AMF hyphae or, with higher precision, of the AMF-specific fatty acid C16:1ω5 (Olsson and Johnson 2005). We estimated the return of investment with respect to nitrogen and phosphorus for each of the two plants, using ^{15}N and ^{33}P as tracers added to the label-hyphal compartment (LHC; Figure 2.1). As a control, we also grew two monocultures and a mixed culture without any AMF inoculation.

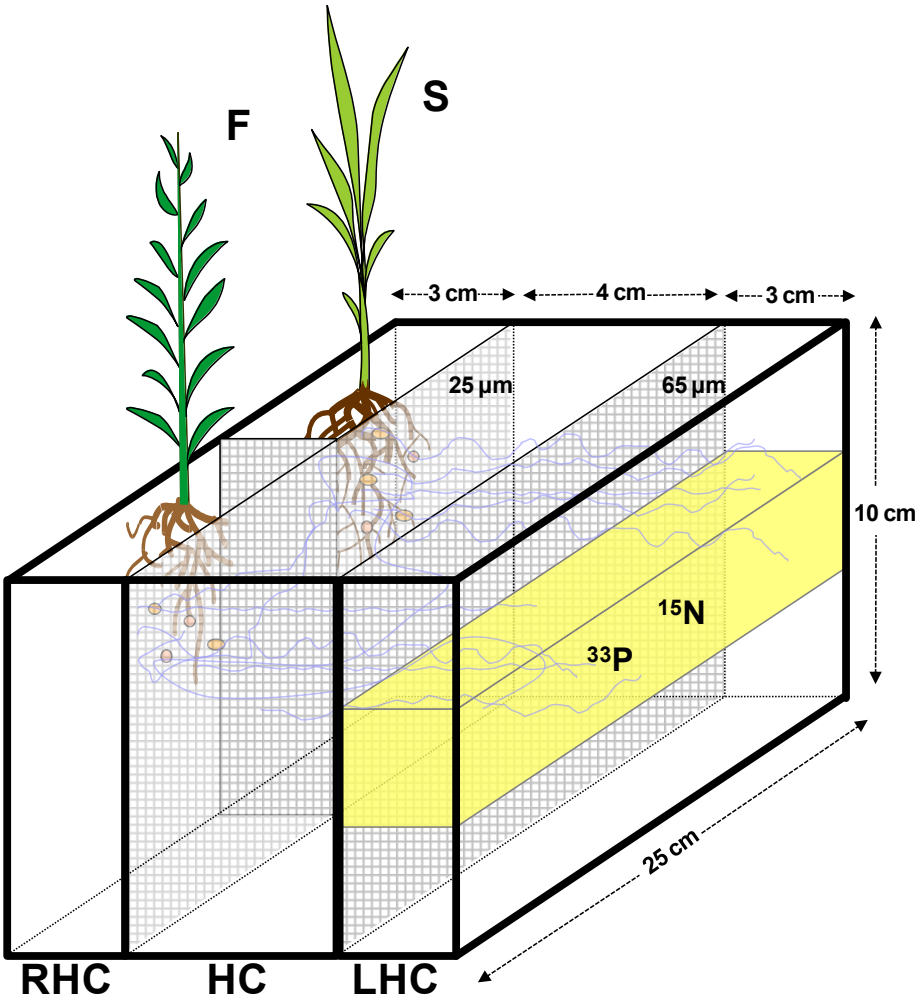

Figure 2.1 Compartmented microcosms to study the role of common mycorrhizal networks in monocultures and mixed culture. Microcosms, consisting of two plant individuals, were set up in compartmented containers subdivided by nylon mesh screens (25 µm and 65 µm, respectively, as indicated). Both types of screens are pervious for fungal hyphae but not for roots and allow the separation into two root-hyphal-compartments (RHC), a hyphal-compartment (HC) and a label-hyphal-compartment (LHC) for supplying ^{15}N- and ^{33}P-labels. The plants used were flax (F) and sorghum (S), either as a pair of conspecific plants (F:F, S:S) as a model of monoculture, or in combination (F:S) as a model of a mixed culture.

2.3 RESULTS

Impact of common mycorrhizal networks on monocultures and mixed culture

A first experiment in the compartmented microcosms (Figure 2.1) demonstrated that in mixed culture with sorghum, flax grew poorly in the absence of AMF. Its growth was significantly enhanced (almost by a factor of three), however, in the presence of a CMN formed by *Glomus intraradices* (Figure 1.2, compare center top and bottom). Growth of sorghum, in contrast, was not significantly affected by the presence or absence of a CMN (Figure 2.2). Comparing the growth performance of mono- versus mixed culture of flax and sorghum in a CMN, respectively was equally striking (bottom part of Figure 2.2): Flax profited substantially (+46% more biomass) from a neighboring sorghum, while sorghum was only marginally, negatively (-7%) affected by the mixed culture growth with flax as neighbor. Thus, the biomass increase of flax did not happen at relevant expense of the neighboring sorghum. Apparently, the two plants had different terms of trade with the CMN of *G. intraradices*, resulting in an overall higher productivity of the mixed culture, compared to the mean of the two monocultures of flax and sorghum (5.97 g ± 0.18 SEM *versus* 5.36 g ± 0.14 SEM, respectively, p-value = 0.039; amounting to 11% overall biomass increase by mixed culturing).

The carbon investment of the two plants into the CMN was quantified through the analysis the carbon isotope composition ($\delta^{13}C$; for definition see materials and methods) of extracted AMF hyphae (Supplemental Figure S2.1A). The hyphal material obtained from the flax monoculture had a $\delta^{13}C$ value of ~27‰, i.e., slightly higher to the $\delta^{13}C$ of the host plant (~33‰). Hyphae from the sorghum monoculture displayed a $\delta^{13}C$ of ~13‰, very close to the value of sorghum plants ($\delta^{13}C$ = ~14‰). Interestingly, the $\delta^{13}C$ of the hyphal material from the mixed culture was also very close to the one of the sorghum monoculture, providing first indication that around 80% of the carbon invested into the CMN originated from sorghum (Supplemental Figure S2.1A).

In the mixed culture, the return of investment in terms of nutrient uptake by the plants, measured as relative uptake of ^{33}P and ^{15}N from the label-hyphal compartment (LHC; Figure 2.1), was similarly unbalanced, but in the opposite sense. In the mixed culture, flax obtained the lion's share of both nutrients, i.e. in the range of ~80%, compared to about ~20% for sorghum (Supplemental Figure S2.1, B and C).

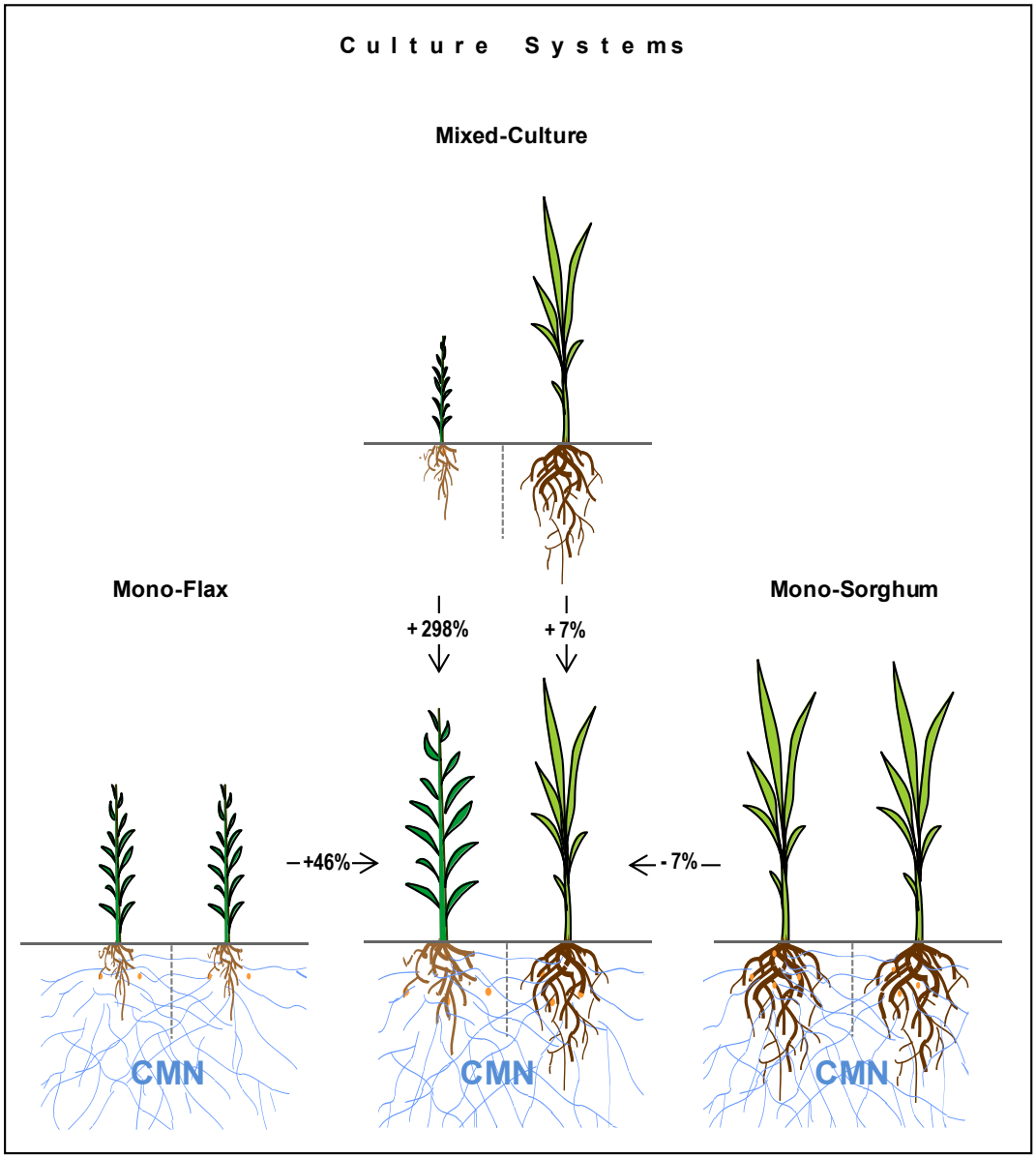

Figure 2.2 Impact of a com-mon mycorrhizal network (CMN) in monocultures and mixed culture. The presence of a CMN of *Glomus intraradices* strongly en-hanced biomass production of flax in mixed culture with sorghum. Sorghum was not significantly affected by the presence of a CMN in the mixed culture with flax. The flax plants grew significantly faster in the flax/sorghum mixed cultures than in the flax monocultures. Conversely, growth of sorghum was only marginally influenced by the culture system.

To confirm and extend these findings, we conducted a second experiment with two different AMF (*G. intraradices* and *G. mosseae*). As in the first experiment, biomass accumulation and P and N content of the flax plants were higher when grown in a CMN together with a neighboring sorghum, irrespective of the fungal species (Figure 2.3, A, C and E). In contrast, the growth performance of sorghum was not affected (Figure 2.3B). As a consequence, the overall productivity in the mixed culture was, again, higher than in the combined monocultures. Despite the absence of any significant growth effect, sorghum also seemed to benefit from the AMF, as indicated by the significant increase in P and N (except N with G.i. in mixed culture) contents (Figure 2.3, D and F). Growth limiting factors at the time of labeling, e.g. constraints of rooting space, might have lead to a surplus or "luxury" carbon as well as a reduced sink strength for soil nutrients.

An AMF-specific fatty acid as biomarker for the plants' carbon investment

In order to quantify the carbon investments into the CMN more precisely, we selectively analyzed the carbon isotopic composition of the AMF specific fatty acid (FA C16:1ω5) in the lipid fraction obtained from the hyphal compartment (HC, Figure 2.1). This way, potential contamination of the hyphal material by non-symbiotic fungi or other microorganisms, can be excluded. Indeed, confirming its use as a marker for AMF, we found FA C16:1ω5 exclusively in the microcosms inoculated with AMF. As expected, the FA C16:1ω5 in the hyphal compartment (HC, Figure 2.1) of the monocultures inoculated with *G. intraradices* or *G. mosseae* displayed similar C isotopic signature as their host plants, confirming that the AMF rely exclusively on the carbon of their symbiotic partners. The fact that the biomarker $\delta^{13}C$ values were consistently lower by ~2‰ than those of the host is likely due to the small but measurable and constant carbon isotope discrimination during carbon transfer from the plants to the lipids of the arbuscular mycorrhizal fungi (Figure 2.4). Remarkably, in the mixed culture (F:S), the $\delta^{13}C$ values for the extraradical mycelium of both *G. intraradices* and *G. mosseae* were much closer to $\delta^{13}C$ of sorghum than to that of flax in monoculture, roughly confirming our initial finding that the carbon invested into the CMN of the mixed culture derived to ~70% from sorghum and only to ~30% from flax, independent of the fungi involved (Figure 2.4).

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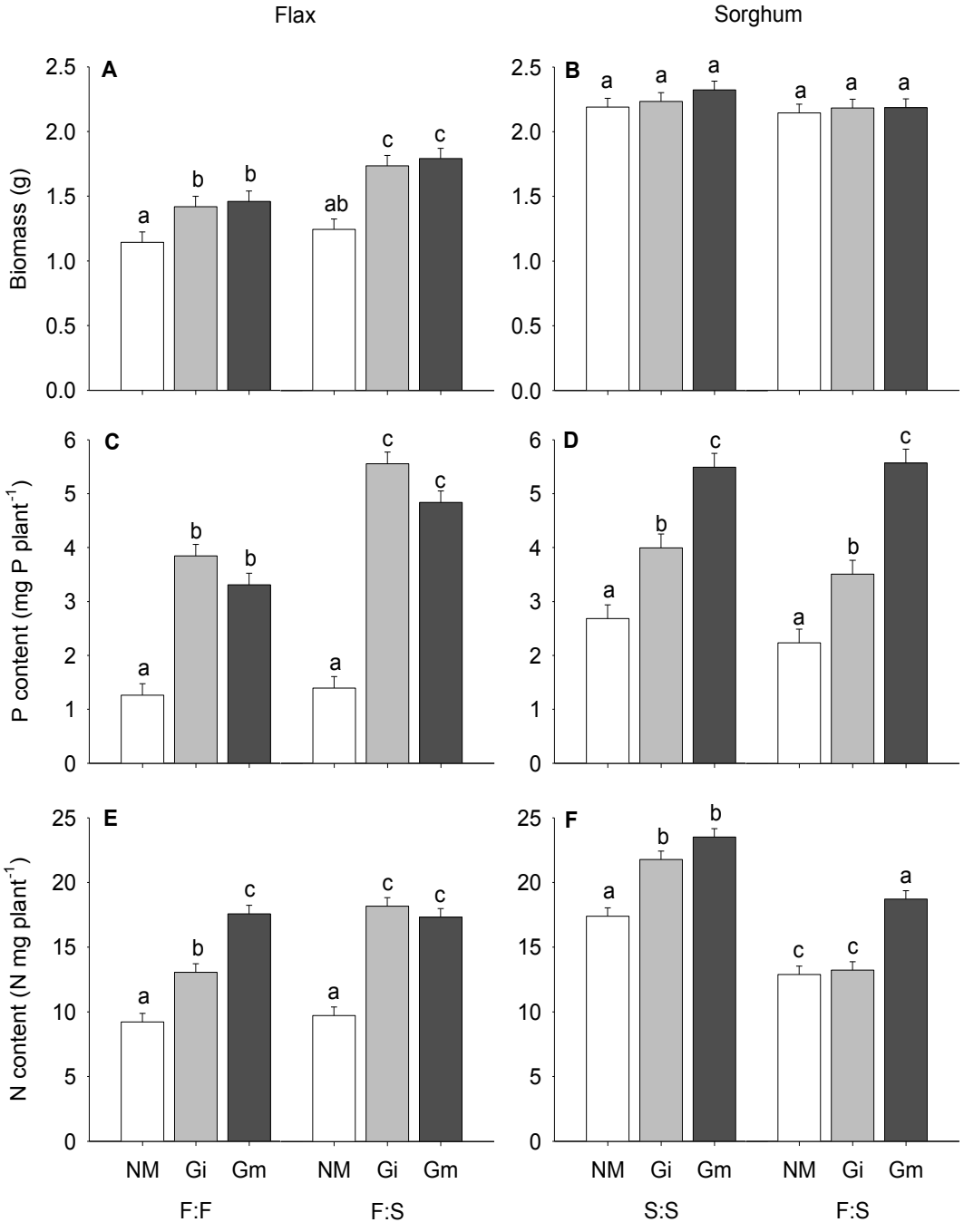

Fig
ure 2.2 Impact of a common mycorrhizal net-work on plant growth performance and nutrient uptake in monocultures or mixed culture. Left side (A, C, E): Performance of flax in monoculture (F:F) or mixed culture (F:S). Right side (B, D, F): Performance of sorghum in monoculture (S:S) or mixed culture (F:S). Data represent mean ± SEM (N=6). Different lowercase letters above bars indicate a significant difference (P≤0.05) among treatments according to planned contrast analysis; mean comparisons were treated separately for both plant species.

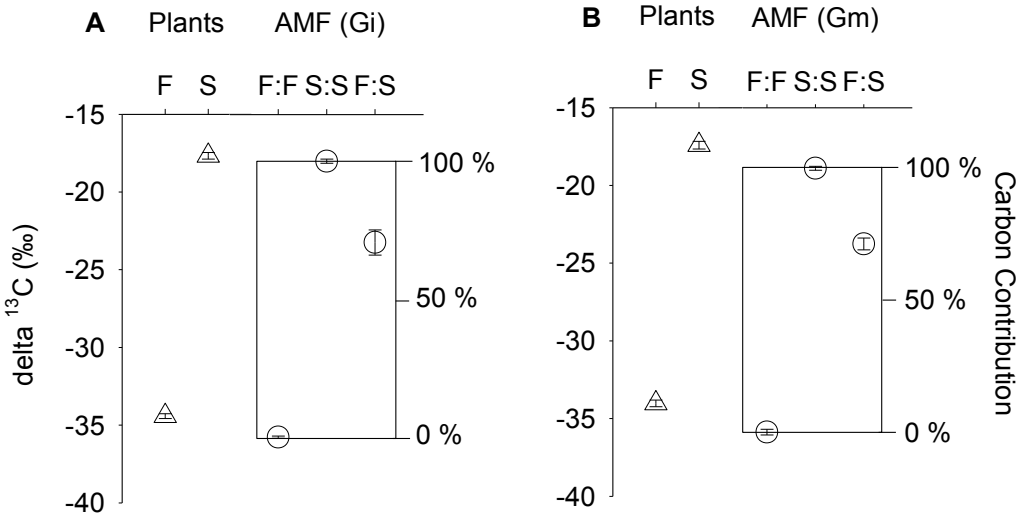

Figure 2.3 Carbon investment of two plants sharing a common mycorrhizal network. δ ^{13}C values of plant tissue (triangles, mean ± SEM, N=36) differ between flax (F, C3-plant, ~34‰) and sorghum (S, C4-plant, ~17‰). The δ ^{13}C values of the AMF-specific FA C16:1ω5 (circles, mean ± SEM, N=6.) were ~2‰ below the values of the corresponding host plants in the hyphal networks of *Glomus intraradices* (A) or *Glomus mosseae* (B) when assessed in monocultures (F:F/S:S). In the mixed culture (F:S), the carbon contribution of sorghum is ~70% in both investigated hyphal networks.

Nutritional benefit gained *via* common mycorrhizal networks

The non-mycorrhizal systems did not take up any ^{33}P and relatively little ^{15}N (Figure 2.5, A and B), indicating that at the time-scale of the experiments, nutrient mobilization due to diffusive processes or massflow did not play any significant role (Figure 2.5, C and D). Thus, virtually all ^{33}P and the bulk of ^{15}N acquired by the plants engaged in the CMN must have come from the fungal partners. Indeed, in monocultures with AMF, both flax and sorghum were able to retrieve substantial amounts of ^{33}P and ^{15}N (Figure 2.5, F:F and S:S). Interestingly, *G. mosseae* delivered about twice as much ^{33}P to sorghum than *G. intraradices* (Figure 2.5B, S:S) whereas similar amounts were delivered to flax by both fungi (Figure 2.5A, F:F) . As a control, we also compared the relative uptake of ^{33}P and ^{15}N for the two plant individuals grown in monoculture. In all cases, nutrient acquisition by the two plants was comparable (Supplemental Figure S2.2, F:F and S:S). When comparing the acquisition of the isotopically labeled nutrients through the CMN in monocultures and mixed culture, we observed marked differences depending on the fungal species involved. With a CMN formed by *G. intraradices,* flax received more than twice as much ^{33}P, and also a little more ^{15}N, in mixed culture than in monoculture (Figure 2.5, A and C, F:S, grey columns).

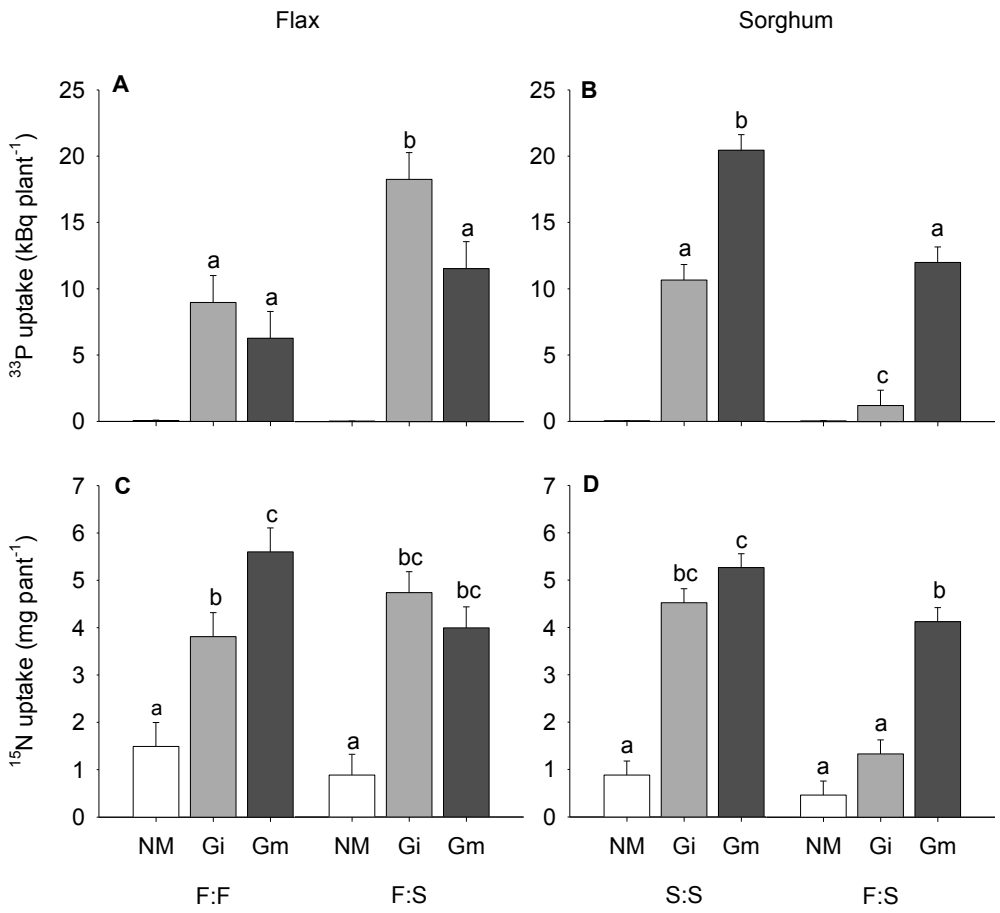

Figure 2.4 CMN-mediated uptake of nitrogen and phosphorus in plants sharing a common mycorrhizal network. Total ^{33}P (A, B) and ^{15}N (C, D) uptake of individual flax (A, C) and sorghum (B, D). Plants were grown in monoculture as pairs of identical plant species (F:F/S:S) or in mixed cultures as pairs of different plant species (F:S), inoculated with a non-mycorrhizal control (NM), with *Glomus irregulare* (Gi), or with *Glomus mosseae* (Gm). Values are means ± SEM (N=6.). Mean comparisons are treated separately for both plant species. Different lowercase letters above bars indicate a significant difference (P≤0.05) among treatments, according to planned contrast analysis.

In contrast, sorghum obtained much less ^{33}P and ^{15}N in mixed culture than in monoculture (Figure 2.5, B and D, grey columns), indicating that flax used the CMN of *G. intraradices* highly efficiently for nutrient uptake, at the expense of sorghum. This corroborates the results of the first experiment (Supplemental Figure S2.1, B and C) and becomes particularly apparent when the data are plotted as relative uptake values (Supplemental Figure S2.2, F:S): 94% of the ^{33}P and 80% of the ^{15}N supplied to the plant pair *via* the CMN was secured by flax. In contrast, with a CMN formed by *G. mosseae,* flax did not benefit significantly from a neighboring sorghum (Figure 2.5, A and C, F:S, black columns). At the same time, sorghum did not suffer intensively from the neighboring flax, although there was still a significantly reduced uptake of nutrients in mixed culture

compared to monoculture (Figure 2.5 B and D, F:S, black columns). In terms of relative uptake, there was no significant difference between flax and sorghum in the mixed culture (Supplemental Figure S2.2). Thus, the respective nutrient return to flax and sorghum strongly differed between the two AMF: Flax was much more efficient than sorghum in exploiting the CMN of *G. intraradices,* whereas in symbiosis with *G. mosseae* the two plants exploited the CMN on equal terms, though sorghum invested much more carbon than flax (Figure 2.4).

Mycorrhizal root colonization and hyphal length density

Both flax and sorghum were well colonized by AMF, with total colonization ranging between 40 and 62 % and arbuscular colonization ranging between 31 and 43% (Supplemental Table S2.2). For flax, colonization was similar in monoculture and mixed culture with both AMF species. As for sorghum, both root colonization and arbuscular colonization were significantly reduced in mixed culture, compared to the monoculture, indicating that sorghum plants were less engaged in the symbiosis in the presence of neighboring flax plants. The extraradical hyphal-length density in the hyphal compartment was slightly higher in sorghum monoculture than in the flax monoculture, although statistically significant only in the case of *G. mosseae* (Supplemental Figure S2.3). In the mixed culture, hyphal-length density was intermediate.

2.4 DISCUSSION

Uneven Terms of Trade in a Common Mycorrhizal Network

Our results (for a graphical synopsis see Figure 2.6) emphasize the importance of the terms of trade within a CMN as a driver for co-existence of mycorrhizal plants in ecosystems. In our mixed-culture experiments, sorghum, as the plant with the higher biomass, consistently provided the bulk of carbon to both tested fungal partners, investing at least twice as much into the CMN as flax. However, the nutritional benefit to the two host plants strongly depended on the fungus involved: In the case of *G. intraradices*, flax might be viewed as a "cheater" on sorghum, acquiring 80-90% of the total labeled N and P provided by the CMN, whereas the acquisition of labeled N and P was more balanced in the case of *G. mosseae* (Figure 2.6). Obviously, in our experiments, carbon investment and nutritional benefit were not tightly linked. This stands in contrast to recent findings where the resource exchange in the symbiosis of plants with AMF appeared to rely on reciprocal “fair-trade” (Javot et al. 2007a, Pietikainen and Kytoviita 2007, Kiers et al. 2011, Fellbaum et al. 2012). At least, with lower levels of root colonization sorghum did express a negative response to the diminished nutritional benefit in mixed culture with flax, so that certain reciprocity of investment and nutritional benefit became also apparent in our system. It has been proposed that the symbiosis between plants and AMF is based on the exchange of “luxury goods” (Kiers and van der Heijden 2006). Hence, CMNs can exist without causing significant additional costs to either partner, especially when the carbon cost is negligible for the main carbon donor. This appeared to be the case for sorghum, which dominated (~60% by biomass weight) in our mixed cultures, or more obviously, for large trees supporting small mycoheterotrophic plants (Courty et al. 2011).

In natural plant communities, the demand for “AMF services” such as soil nutrient acquisition and, *vice versa*, the availability of “luxury goods” such as a surplus of carbon, is expected to dynamically change for the different plants, depending on their strategies to respond to environmental cues and their specific life-history traits with consecutive phases of vegetative growth, maturation, senescence etc. Thus, CMNs supposedly function as dynamic “marketplaces” in biodiverse ecosystems, where the symbionts involved and apparently organized in networks of plant-AMF assemblages (Montesinos-

Navarro et al. 2012), can offer temporary "luxury goods" in exchange for more limited resources. As logic consequence, these trades can only weakly be reciprocal, but dependent on transient sink strengths and the efficiency of exchanges at the various symbiotic interfaces, that can differ for different plant-fungus combinations (Klironomos 2003, Helgason et al. 2007). This is evident in our mixed cultures where sorghum, in return for a similar expenditure of C received much more phosphorus from *G. mosseae* than from *G. intraradices* whilst for flax it was inverse. This difference in functional compatibility between host plants and fungal partners was also displayed in the monocultures, where sorghum acquired more phosphorus from *G. mosseae* than from *G. intraradices* whilst flax acquired marginally more phosphorus from *G. intraradices*. With regard to our findings with the mixed cultures, it would be challenging to monitor the trading of the two plants in the CMN over their whole life cycle and/or under changing sink-source relationships, elicited for instance by a change of the light regimes or by leaf clipping. To this end, the $\delta^{13}C$ values of the respired CO_2 in the hyphal compartment could be monitored.

Sharing luxury goods maximizes productivity

Our experimental data clearly demonstrate that an unbalanced use of the CMN not only can increase the growth of an individual plant such as flax, but it also can increase the productivity of our two-plant model ecosystem by sharing the benefit of a luxury good (carbon provided by sorghum) between sorghum and flax (Kiers and van der Heijden 2006). There are other possibilities for plants of different functional groups to jointly profit from the CMN as a common good, e.g. by taking advantage of the proficiency of legumes to fix nitrogen in symbiosis with rhizobia (Jalonen et al. 2009) or the capacity to lift water by deep-rooting (Egerton-Warburton et al. 2007). By complementary use of different resources, in biodiverse ecosystems, plants may cooperatively maintain CMNs without causing exorbitant costs to any of the partners joined in the network. This may explain how the presence of AMF promotes productivity and diversity of plant communities (van der Heijden et al. 1998b).

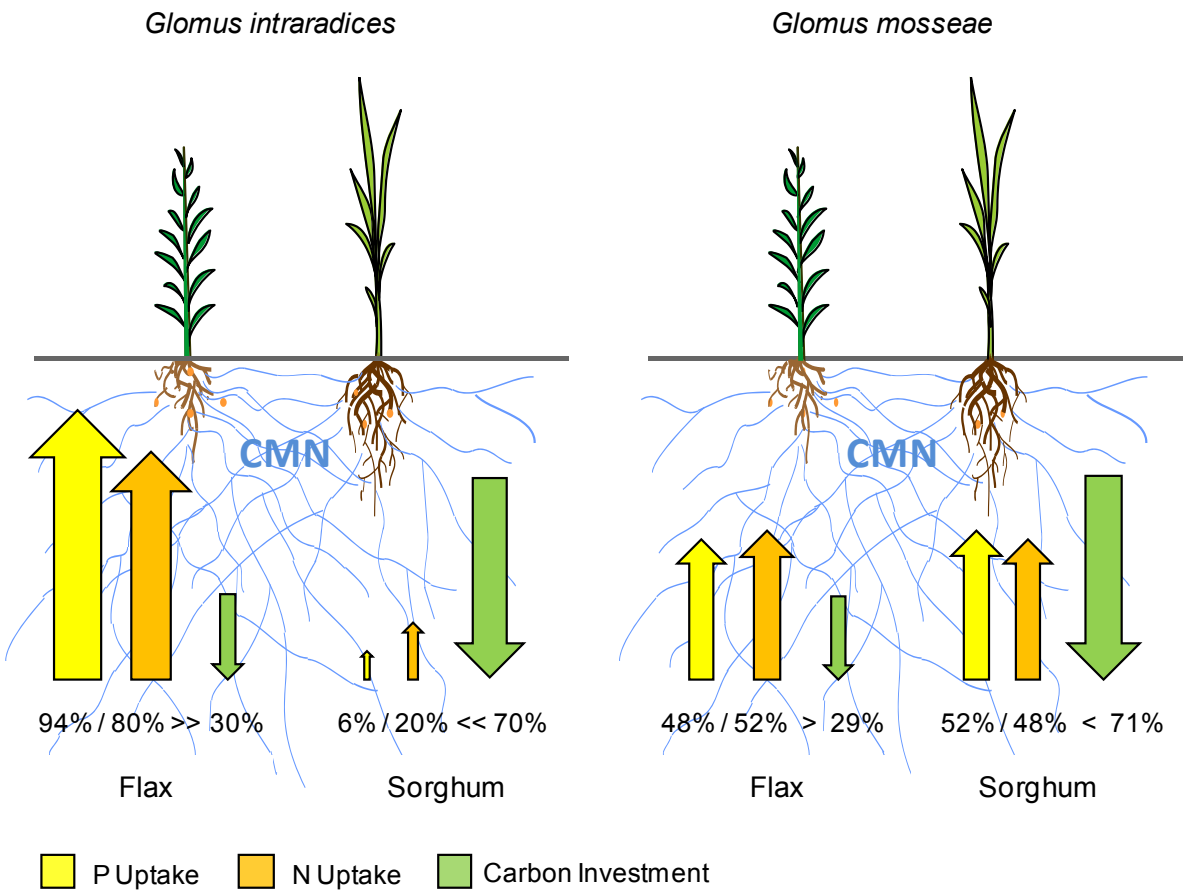

Figure 2.5 Terms of trade in common mycorrhizal networks (CMN) between flax and sorghum, formed by *G. intraradices* and *G. mosseae*. In this scheme, the carbon investment of the plants is depicted by a green arrow. In both CMNs, sorghum invested more than twice as much than flax in terms of carbon. The return, in form of the nutrients P and N, is illustrated by the yellow and orange arrows, respectively. In the CMN formed by *G. intraradices,* the return was extremely uneven; flax obtained 80-94% of the nutrients delivered by the CMN, and sorghum, the main investor, only 6-20%. In the CMN formed by *G. mosseae*, both flax and sorghum received an approximately equal share of the nutrients delivered by the CMN, but since flax invested less than half as much carbon compared to sorghum, it still benefited from its neighbor.

2.5 CONCLUSION

Traditional agricultural systems, which have emerged over millennia globally at multiple locations, usualy, display a high biodiversity engendered by meticulously planned mixed culturing practices, including agro-forestry (Jalonen et al. 2009, Bainard et al. 2011). Such biodiverse agroecosystems – for example the diverse cereal-legume intercropping systems traditionally used in Asia (Li et al. 2007) and in Africa (Snapp et al. 2010) - have been shown repeatedly to be more efficient and often also more productive than conventional monocropping systems (Hauggaard-Nielsen and Jensen 2005, Perfecto and Vandermeer 2010, Hinsinger et al. 2011). This is currently ascribed to effects such as complementary resource use, resilience and yield stability under stress conditions or pest and pathogen control by facilitation of antagonists (Altieri 2002). We propose that, in addition, such biodiverse agroecosystems were unwittingly developed by resource-poor farmers to make maximal use of CMNs. A revival of favorable intercropping systems, considering the extensive experience and knowledge of indigenous communities in combination with ongoing efforts to better comprehend the intricacies of the CMN's, may help to boost productivity in a sustainable way and, thus, contribute to satisfy the increasing global demand for food (Godfray et al. 2010).

2.6 MATERIAL AND METHODS

Organisms and microcosms

The two host plants used were flax (*Linum usitatissimum cv*. Agatha) and sorghum (*Sorghum bicolor cv.* Pant Chari-5). The two fungal partners, both of the genus *Glomus* (Phylum: Glomeromycota), were *Glomus intraradices*, strain "*TERI commercial*" (Mathimaran et al. 2008) and *Glomus mosseae*, strain *ISCB 22*, both kept in our fungal strain collection.

Pairs of plants were planted into microcosms (25 x 10 x 10 cm^3) with four compartments, as illustrated in Figure 2.1, and grown under controlled conditions (16 h light (220 µE m^{-2} s^{-1}) at 25 ºC and 8 h dark at 20 ºC, constant relative aerial humidity of 65%). The root-hyphal and hyphal compartments (RHC and HC) were separated by a 25 µm nylon mesh (Lanz-Anliker AG, Rohrbach, Switzerland), sandwiched between two 500 µm fiber-glass meshes (Tesa AG, Hamburg, Germany), allowing fungal hyphae, but not plant roots, to enter the HC. The HC and label-hyphal compartments (LHC) were separated by a 65 µm nylon mesh. All compartments were filled with sterile (120° C, 20 min) growth substrate consisting of a mixture of Terragreen (American aluminium oxide, oil dry US special, type III R, 0.125 mm; Lobbe Umwelttechnik, Iserlohn, Germany), sand (Quartz sand d`Alsace, 0.125 – 0.25 mm, Kaltenhouse, France) and Loess from a local site (5:4:1 [wt/wt/wt]). The substrate had the following chemical properties; pH (H_2O) ~ 6, organic carbon < 0.5 g C kg^{-1}, P_2O_5, (Na-acetate) = 3 mg kg^{-1}, P_2O_5, (double lactate) = 35 mg kg^{-1}, K_2O (Na-acetate) = 45 mg kg^{-1}, K_2O (double lactate) = 47 mg kg^{-1}, clay content < 5 %. The chemical parameters were measured in the laboratory of F.M. Balzer, Wetter-Amönau, Germany (http://www.labor-balzer.de).The RHCs were inoculated with a 2 g (approx. 100 spores/ compartment) inoculum of one of the *Glomus* strains, or with 2 g sterilized (120° C, 20 min) inocula as non-mycorrhizal control. In addition, the RHCs received 10 ml of a microbial wash to equalize microbial communities (Koide and Elliott 1989). This wash was prepared by wet sieving 100 g of each inoculum through a 32 µm sieve and a paper filter (FS 14 ½, Schleicher & Schuell) yielding a final volume of 1 L. The LHC (Figure 2.1) was filled with 325 g of growth substrate including a 100 g layer labeled with 500 kBq $^{33}PO_4^{3-}$ (Hartmann Analytic, Braunschweig, Germany) and^{15}N (100 mg $^{15}NH_4{}^{15}NO_3$, (Cambrigde Isotopes Laboratories, Inc., Andover, MA, USA)) placed in the

middle of the LHC. This compartment was attached to the microcosm after 8 weeks of cultivation. The voluminous HC provided a diffusive barrier for ^{33}P and ^{15}N isotopes towards the RHC, and, thus, minimized direct uptake by root hairs that may have reached into the HC by passing the nylon net during the experiment.

The microcosms were watered with distilled water twice a week in the RHCs and HC, and thereby adjusted to equal soil water content of 90% field capacity by weighing. In addition, every week during the first eight weeks of cultivation, the RHC was amended with 8 ml of a P-free Hoagland Solution (Gamborg and Wetter 1975, Zabinski et al. 2002).

Experimental design

In each of the two RHCs, one single plant was grown, yielding microcosms with "monocultures" (a pair of identical plants) or a "mixed culture" (one flax and one sorghum plant). In the preliminary experiment, the plants were inoculated either with *G. intraradices* (Gi) or with the sterilized control inoculum (NM). In the second experiment, growth experiments were also conducted with *G. mosseae* (Gm). The microcosms were harvested after 12 weeks of growth.

Plant growth performance and symbiotic interaction

Roots were washed thoroughly, excess moisture was removed, and fresh weight was determined. Two subsamples were weighed, one of which was then used for determination of root dry weight. The other aliquot was cleared using a 10% KOH solution, and stained in Trypan Blue for mycorrhizal-structure identification inside the root (Phillips and Hayman 1970). The percentage of root length occupied by hyphae, arbuscules and vesicles was estimated for each sub-sample by a modified line intersection method (McGonigle et al. 1990). A minimum of 50 line intersection per root sample were scored for AMF. Shoot and root samples were dried for 24h at 105 ºC and weighed separately; the sum corresponds to the "total biomass" indicated in the figures. Dried shoots and roots were ground at 30 Hz in a mixer mill (MM2224, Retsch, Haan, Germany). Aliquots of 2 mg were weighed in for elemental analyses. Nitrogen and carbon concentrations were determined using an ANCA elemental analyzer/mass spectrometer

(Europa Scientific Ltd., Crewe, UK). P concentration of shoots and roots was measured using the molybdate blue method on a Shimadzu UV-160 spectrophotometer (Shimadzu Biotech, Duisburg, Germany) after acid digestion (Murphy and Riley 1962). The substrate of the HC was stored at -20 ºC. A subsample of 50 g was used for hyphal length density measurements, determined by the grid-line-intersection method (Jakobsen et al. 1992).

Nutrient gain of the mycorrhizal network

Plant ^{33}P contents were measured using a Packard 2000 liquid scintillation counter (Hewlett-Packard, Waldbronn, Germany). The ^{15}N content of plants was analyzed with an ANCA mass spectrometer (Europe Scientific Ltd., Crewe, UK). Relative ^{33}P and ^{15}N uptake were calculated by dividing uptake of individual plants by the total uptake of both plants of the microcosm.

Carbon contribution to mycorrhizal network

The carbon isotope composition of plant shoot and roots and of hyphal biomass was determined using an ANCA IRMS. Extraradical hyphae were extracted from the HC by a wet sieving method (Johansen et al. 1996). The recovered hyphae were dried in a DNA-Speed Vac (Savant) prior to bulk mass spectrometric analysis. For compound-specific analyses of the AMF-specific fatty acid (FA) C16:1ω5, lipid extraction was carried out according to previously described methods (Elvert et al. 2003, Niemann et al. 2005). Briefly, total lipid extracts were obtained by suspending and sonicating 25 g of freeze-dried substrate of the HC in organic solvents of decreasing polarity. Internal standards (*n*-nonadecanol and *n*-nonadecanoic acid) of known concentration and carbon isotopic composition were added prior to extraction. Total lipid extracts were saponified with a methanolic KOH-solution (6%). After extraction of the neutral fraction from this mixture, FAs were methylated using a boron triflouride solution (14% BF3 in methanol), yielding FA methyl esters. Double bond positions of monounsaturated FAs were determined by analyzing the dimethyl disulfide adducts of FAs (Moss and Lambertfair 1989). The carbon isotopic composition of FA C16:1ω5 was determined by GC-IRMS. All stable

carbon isotope ratios presented here are reported in the conventional δ -notation with respect to the V-PDB (Vienna Pee Dee Belemnite) standard. The relative carbon contribution (RCC) of sorghum to the hyphal network in the mixed culture was calculated on the basis of the mixing model with two endmembers as follows: RCC= [δ^{13}C F:F - δ^{13}C F:S / δ^{13}C F:F - δ^{13}C S:S] (Peterson and Fry 1987).

Statistical analysis

Experiment 1 was set up in a randomized block design where each treatment was replicated four times. Mean comparison among treatments were performed by independent paired t-tests for dry weight and relative uptake of ^{33}P and ^{15}N of the two individual plants.

Experiment 2 was set up in a randomized block design including two temporal blocks with a time lag of 4 weeks. Each block contained three replicates, with a resulting total of 6 replicates per treatment. An analysis of variance (ANOVA) was performed on the total biomass, on the P and N content, and on the total and arbuscular colonization for each plant species separately, where the two latter parameters were arcsine-transformed to fit the assumption of normal distribution. The ANOVA was based on the three factors culture system (with two levels), AMF (with three levels) and block (with two levels). Pairwise comparisons between the treatments were done with planned contrast analysis. Independent paired t-tests were performed to analyze whether means of relative uptake of ^{33}P and ^{15}N of the two individual plants differed significantly from each other. An ANOVA with the factors treatment (9 levels) and block (two levels) was executed on the fungal parameter hyphal length density. A probability of $P \leq 0.05$ was considered as representing a significant difference.

Acknowledgments

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2.7 SUPPLEMENTAL MATERIAL

Supplemental Table S2.1 Dry weight of flax and sorghum, measured after harvest in the different culture systems used in experiment 1

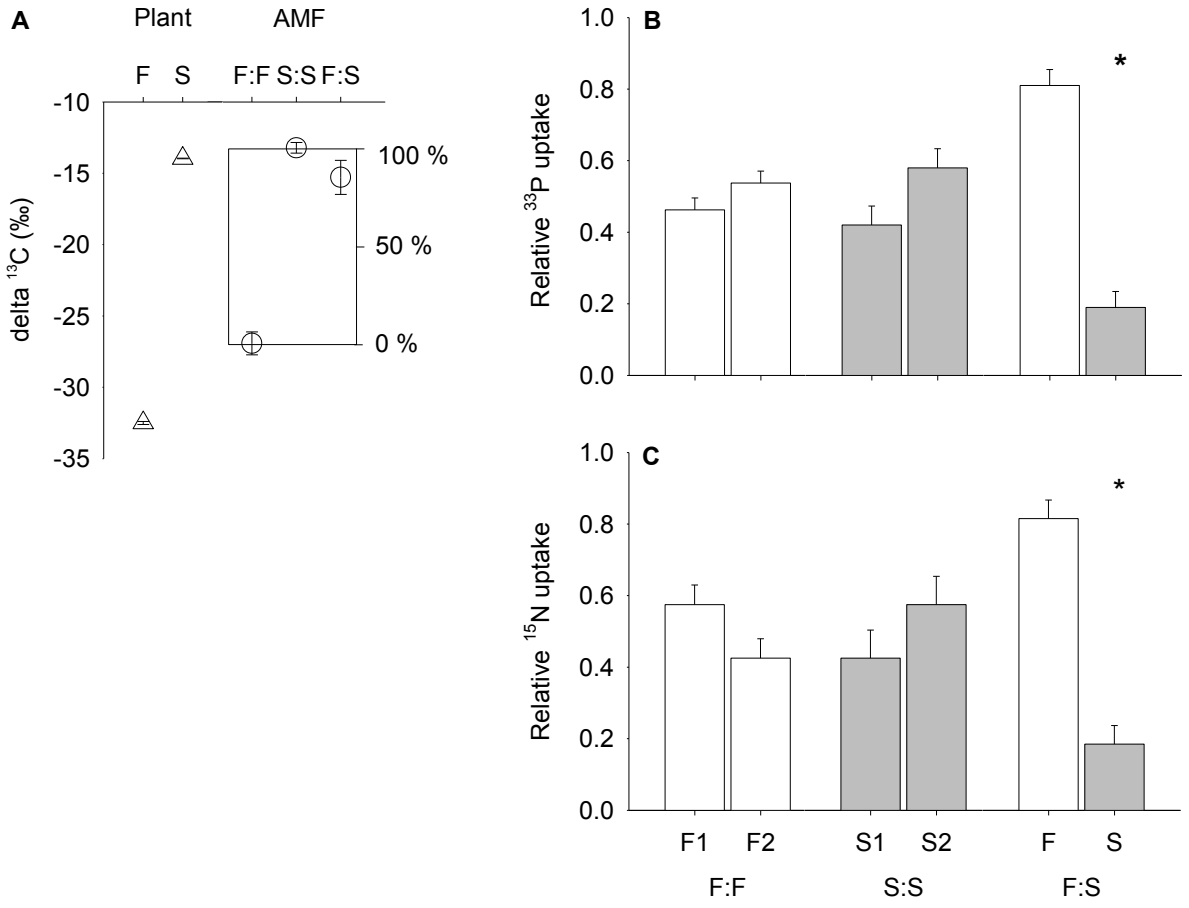
	Flax		Sorghum	
	dry weight (g)	p-value	dry weight (g)	p-value
Mixed -CMN	0.73 ± 0.13	< 0.001	2.89 ± 0.22	0.466
Mixed +CMN	2.89 ± 0.18		3.08 ± 0.09	
Mono +CMN	1.97 ± 0.15	0.019	3.39 ± 0.06	0.033
Mixed +CMN	2.89 ± 0.18		3.08 ± 0.09	

This table provides the raw data for Figure 2.2. Plants were grown in the presence and absence of a common mycorrhizal network (CMN) in mixed culture and in monocultures, or mixed culture in presence of a CMN. The CMN was built by *G. intraradices*. Values represent means ± SEM (N=4). Significance was calculated by independent paired t-tests, p-values are shown for each comparison of means. To improve legibility, the data for "mixed + CMN" are shown twice, at the top and at the bottom of the table.

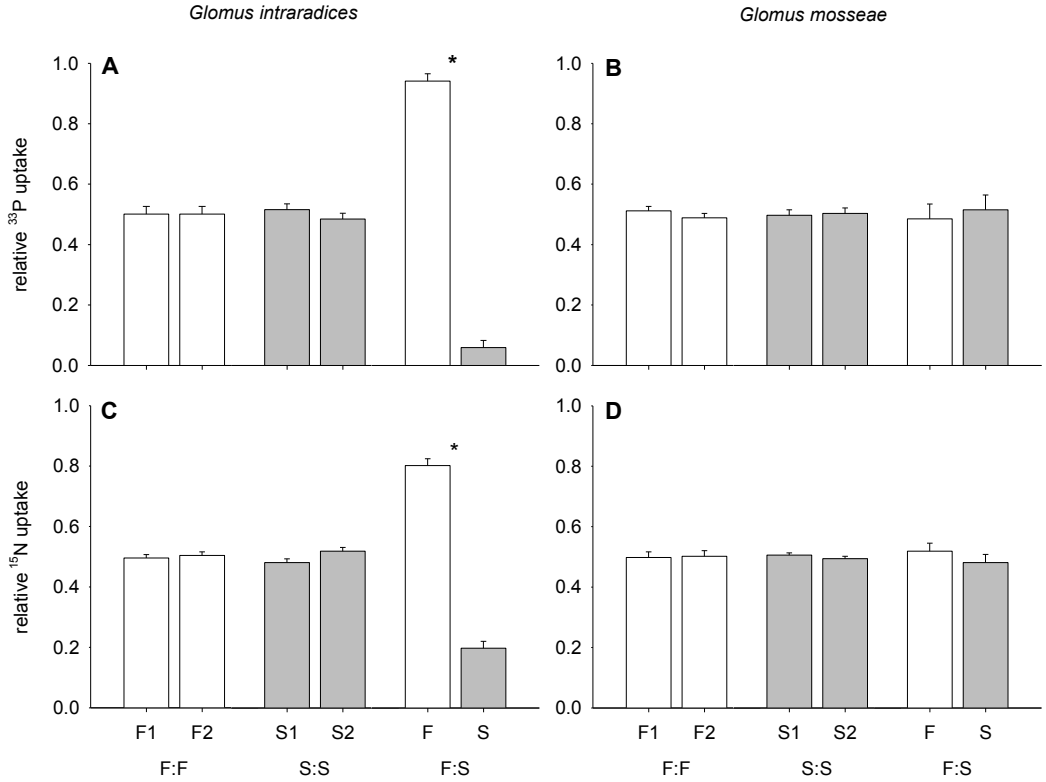
Supplemental Table S2.2 Total and arbuscular root colonization of flax and sorghum.

Plant Species	Culture System	AMF	Total Colonization (%)	Arbuscular Colonization (%)
Flax	Mono	NM	2 ± 0.8	0 ± 0.0
		Gi	44 ± 2.2[a]	35 ± 2.3[a]
		Gm	39 ± 2.2[a]	31 ± 2.5[a]
	Mixed	NM	1 ± 0.8	0 ± 0.0
		Gi	42 ± 5.9[a]	36 ± 5.5[a]
		Gm	42 ± 2[a]	37 ± 2.2[a]
Sorghum	Mono	NM	2 ± 0.5	0 ± 0.0
		Gi	56 ± 3.2[a]	44 ± 3.5[a]
		Gm	62 ± 4.6[a]	53 ± 4.8[b]
	Mixed	NM	3 ± 1.4	0 ± 0.0
		Gi	40 ± 3.8[b]	31 ± 3.7[c]
		Gm	42 ± 2.5[b]	33 ± 3.1[c]

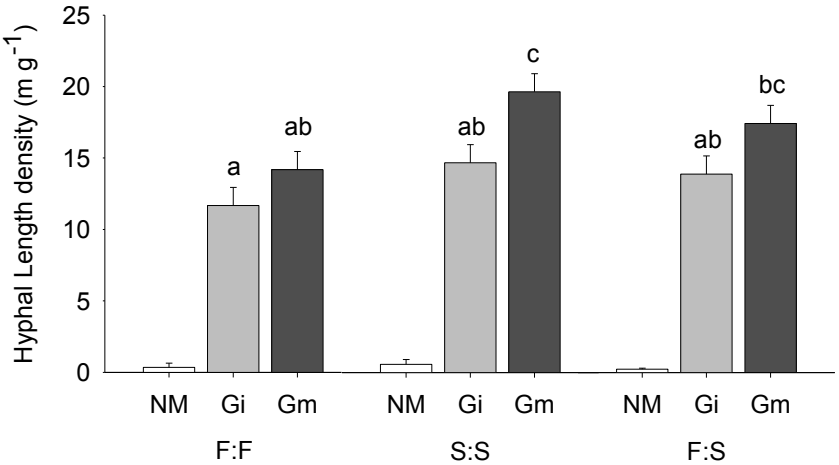
Percentage of root length colonized by AMF structures (total colonization) and by arbuscules (arbuscular colonization) on the harvest day (12 weeks). Values represent means ± SEM (N=6) of colonization of individual host plants in mono-cultures and mixed cultures in the absence of mycorrhizal fungi (NM), and with *Glomus intraradices* (Gi) or *Glomus mosseae* (Gi). Mean comparisons are treated separately for both plant species and parameters. Different lowercase letters indicate a significant difference among treatments, according to planned contrast analysis. The non-mycorrhizal treatment (NM) was excluded from analysis.



Supplemental Figure S2.1 Carbon investment and nutrient return for two host plants sharing a common mycorrhizal network of *Glomus intraradices*. (A) Carbon investment was estimated on the basis of the bulk $\delta^{13}C$ values of fungal hyphae in the hyphal compartment. $\delta^{13}C$ values of plant tissue (triangles, mean ± SEM, N=16) differ between flax (F, C3-plant; ~33‰) and sorghum (S, C4-plant; ~14‰). $\delta^{13}C$ values of extracted AMF hyphae (circles, mean ± SEM, N=4) of the two monocultures (F:F/S:S) were similar to the values of the corresponding host plants. In the mixed culture, the value was very close to the value of AMF hyphae in the sorghum monoculture, indicating that sorghum delivered almost all carbon to the common mycorrhizal network. (B, C) Nutrient return shown as relative uptake of labeled nutrients (^{33}P and ^{15}N), calculated by dividing labeled nutrient uptake of plant individuals by the total uptake per microcosm. Stars above bars indicate a significant difference ($P \leq 0.05$) among neighboring plant species, according to the student's t-test.



Supplemental Figure S2.2 Nutrient return for two plants sharing a common mycorrhizal network. Relative ^{33}P (a, b) and ^{15}N (c, d) uptake of flax (F) and sorghum (S) inoculated either with *Glomus intraradices* (a, c) or with *Glomus mosseae* (b, d). Host plants were planted as monoculture (F:F/S:S) or mixed culture (F:S). Relative uptakes were calculated by dividing labeled nutrient uptake of plant individuals by the total uptake per microcosm. Values represent means ± SEM (N=6). Stars above bars indicate a significant difference (P≤0.05) among neighboring plant species, according to the student's t-test.



Supplemental Figure S2.3 Hyphal length density in the hyphal compartment of monocultures and mixed cultures in the absence of mycorrhizal fungi (NM), and with *Glomus intraradices* (Gi) or *Glomus mosseae* (Gi). Host plants were grown in monoculture as pairs of identical plant species (F:F/S:S) or in mixed cultures as pairs of different plant species (F:S). Values represent means ± SEM (N=6). Different lowercase letters above bars indicate a significant difference (P≤0.05) among treatments, according to LSD test..

3 EXPRESSION OF PHOSPHATE TRANSPORTER GENES IN *SORGHUM* AND *LINUM*: DEVELOPMENTAL ASPECTS AND REGULATION IN A COMMON MYCORRHIZAL NETWORK

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3.1 ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are important plant symbionts, trading mineral nutrients beyond the reach of roots, in particular phosphate, in exchange to their hosts' photosynthetic products. Surprisingly, in a mixed culture between flax (*Linum usitatissiumum*) and sorghum (*Sorghum bicolor*; Walder et al. 2012), flax took up much more of the phosphate delivered by the CMN than sorghum, although sorghum invested much more carbon into the CMN than flax. Is this difference in phosphorus uptake due to differential regulation of phosphate transporters in the two host plants? To examine this question, a baseline was first established by identifying all eleven phosphate transporters of the phosphate transporter family Pht1 in sorghum and studying their expression in different tissues of field-grown plants. While some of them were not expressed in stems, leaves or flower organs, most of them were well expressed in roots; two of them, *SbPT10* and *SbPT11*, were expressed in the roots exclusively in the presence of AMF. We also identified two mycorrhiza-inducible Pht1 transporters in flax. When expression of the mycorrhiza-inducible phosphate transporters was studied in monocultures or mixed cultures of flax and sorghum, it turned out that the expression of AM-inducible *Pht1* genes was only weakly related to mycorrhizal P uptake, but that it was differentially regulated depending on the fungal partner and the co-cultured plant. We conclude that in both sorghum and flax, expression of AM-inducible Pht1 transporters is initiated by arbuscule formation, but that the differential phosphate delivery by the CMN is not directly dependent on differential *Pht1* gene expression in the two host plants.

3.2 INTRODUCTION

Phosphorus (P) is an essential macronutrient playing a central role in developmental and metabolic processes in plants, and one of the most important growth-limiting factors in many natural and agro-ecosystems (Marschner 1995). The primary source of P for plants is inorganic orthophosphate (P_i). P_i concentrations in the soil solution are very low (1-10 µM) and rapidly drop to sub-micromolar levels at the root-soil interface, where a narrow depletion zone is generated because of the highly efficient P_i uptake systems of the roots, combined with the extremely slow diffusion of P_i in the soil solution (Hinsinger et al. 2005, Richardson et al. 2009). To overcome this limitation, P fertilizers are used extensively in agriculture. However, readily available P may be exhausted at the end of this century; therefore, it is a major challenge for plant research to increase P acquisition from the soil in a sustainable way (Vance et al. 2003, Cordell et al. 2009).

Since cellular P_i concentrations are in the millimolar range, direct P_i uptake from the soil solution requires an energy dependent transport system; in the plant root, this function is fulfilled by the P_i/H^+ symporters belonging to the *Pht1* gene family, which are fueled by the protein gradient established at the plasma membrane (Rausch and Bucher 2002). Indeed, many *Pht1* genes are strongly expressed in the rhizodermal cells, including roots hairs and in cortical root cells, indicating a role in direct P_i uptake from soil (Daram et al. 1998, Chiou et al. 2001, Ai et al. 2009). But expression has also been observed in leaves and inflorescences, indicating additional roles of Pht1 transporters such as remobilization of P_i from leaves (Rae et al. 2003), or P_i uptake in the elongating pollen tube (Mudge et al. 2002). The expression of the *Pht1* genes was often found to be upregulated when plants were P limited (Bucher 2007).

In natural ecosystems, plants use a series of adaptions to increase the acquisition of poorly available P, of which the symbiosis with arbuscular mycorrhizal fungi (AMF) is one of the most important. AMF are soil fungi of the phylum Glomeromycota forming symbiotic association with about 80% of terrestrial plants (Parniske 2008). AMF are not host specific; as a consequence, they may form common mycorrhizal networks (CMNs), colonizing simultaneously several plants from the same or different species (Bever et al. 2010). For mycorrhizal plants, AMF play a crucial role in P_i acquisition, accounting for up to 90% of their P requirements (van der Heijden et al. 2008). AMF, with their extraradical hyphae, act as functional extensions of the root system and can access

nutrients such as P_i much beyond the depletion zone (Richardson et al. 2009). In CMNs, some plants may increase their access to P_i by exploiting the CMN more efficiently than the interconnected plants (Zabinski et al. 2002, Walder et al. 2012).

Plants engaged in a the arbuscular-mycorrhizal (AM) symbiosis can absorb P_i through two different pathways; (i) directly *via* the rhizodermis, including the root hairs, through high-affinity plant Pht1 transporters, as described above, and (ii) indirectly *via* the AM symbiosis. The indirect mycorrhizal pathway begins with P_i uptake by high-affinity fungal Pht1 transporters expressed in the extraradical hyphae (Smith and Smith 2011). P_i is then translocated towards the roots and released, by unknown mechanisms, to the periarbuscular space in the arbuscules. There, it is taken up across the plant's periarbuscular membrane, most likely by AMF-inducible Pht1 transporters of the plant (Smith and Smith 2011). Such AMF-inducible P_i transporters have been identified in many plant species of monocots and dicots (Javot et al. 2007b), including perennial trees (Loth-Pereda et al. 2011). They all belong to the family of Pht1 transporters but cluster in two different subgroups, named subfamily I and III (Bucher 2007). Most members of subfamily I are only expressed in arbuscule-containing cortical cells during AM symbiosis, as revealed by immunolocalization (Harrison et al. 2002, Javot et al. 2007a, Tamura et al. 2012). AM-induced *Pht1* genes of subfamily III were found to be expressed more generally in plant roots, but specifically induced in the cortical cells during AM symbiosis (Rausch et al. 2001, Maeda et al. 2006). Studies of mutants with reduced expression of these two types of AMF-inducible *Pht1* genes revealed that both were important for symbiotic P_i uptake (Maeda et al. 2006, Javot et al. 2007a). Recent investigations on the promoter region of AM-inducible *Pht1* genes revealed that at least two cis-regulatory elements, the mycorrhiza transcription factor binding sequence (MYCS) and P1BS, mediate the transcriptional activation of these P_i transporters (Chen et al. 2011).

The study presented here is based on our recent finding that flax (*Linum usitatissiumum*) and sorghum *(Sorghum bicolor*), connected by a CMN, had rather different terms of trade with their AM symbiotic partners (Walder et al. 2012). In particular, flax took up more than ten times more of the phosphate delivered by the CMN than sorghum, although sorghum invested much more carbon into the CMN than flax. Was this difference in phosphorus uptake due to differential regulation of phosphate transporters in the two host plants?

3.3 MATERIALS AND METHODS

Plant material

For studies of gene expression in different sorghum tissues, three fully grown sorghum plants were harvested in a field site located in Northeastern France (47°62'N, 7°52'E) in September 2011. For each plant, three subsamples (about 100 mg) of fresh roots, stems, shoots, pistils and stamina were snap frozen and lyophilized for further gene expression analysis. Additionally, from each of the three plants, four subsamples (about 100 mg) of fresh roots were snap frozen and stored at -80°C for further gene expression analysis and molecular identification of the arbuscular mycorrhizal species according to previously published methods (Courty et al. 2011). A fifth root subsample from each plant was cleared using a 10% KOH solution, and stained with Trypan Blue to identify mycorrhizal structures inside the root (Phillips and Hayman 1970). For studies of gene expression in sorghum roots under different conditions of mycorrhization and phosphate availability, seeds of cultivar Plant Chari 5 were surface sterilized (5min 70% EtOH, 10min in 2.5% KClO), rinsed with sterile deionized water and soaked overnight. Seeds were pre-germinated on autoclaved sand (Quartz sand of Alsace, 0.125 – 0.25 mm, Kaltenhouse, France) for 72h in the dark at room temperature. To establish AM symbiosis, pre-germinated seedlings were planted individually in 350ml pots containing a mixture of sand, acid washed Terragreen (American aluminium oxide, oil dry USspecial, type III R, 0.125 mm; Lobbe Umwelttechnik, Iserlohn, Germany) and Loess from a local site (4:1:1 [wt/wt/wt]) and were inoculated at the same time with about 100 spores (2g). The two fungal strains, *Glomus intraradices* TERI commercial (Mathimaran et al. 2008) and *G. mosseae* ISCB 22 (Botanical Institute, Basel, Switzerland), were propagated as previously described in detail (Oehl et al. 2003). For the non-mycorrhizal plants, the same amount of autoclaved (120°, 20 min) inoculum was added. All pots received 5 ml of a microbial wash to normalize microbial communities (Koide and Elliott 1989). The microbial wash was prepared by wet sieving 100 g of each inoculum through a 32-µm sieve and a paper filter (FS 14 ½, Schleicher&Schuell) yielding a final volume of 1 L. The pots were watered weekly with deionized water. From the first week, 8ml of full or P-free Hoagland Solution (with or without the component $NH_4H_2PO_4$) was applied weekly (Zabinski et al. 2002). Plants were grown under controlled condition in a greenhouse. Night temperature was set to 18°C and day temperatures varied between 23 and 30°C

depending on the weather conditions outside. Additional lighting was provided with high-pressure mercury vapor lamps (Philips HPL-N, 400 W) to a day length of 16 h per day. The experiments were set up in a randomized block design with four replicates yielding in a total of 24 pots. For the studies on the gene expression in monoculture or mixed culture, the plant material was taken from the microcosms described previously, in which sorghum and flax plants were raised in separate compartments, connected by a common hyphal compartment (Walder et al. 2012).

Analysis of DNA

DNA from sorghum roots was extracted and analyzed according to a previously described method (Courty et al. 2011). Briefly, after isolation of DNA with the NucleoSpin tissue KS kit (Macherey-Nagel), the ITS region of nuclear ribosomal DNA was amplified on a T3 thermocycler (Biometra) and the amplified fragments were then sub-cloned using the TOPO-TA cloning kit (Invitrogen). Sequences were manually corrected using Sequencher 4.2 (Gene Codes). To identify fungal species, BLASTN searches were carried out against the sequence databases at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Root colonization by arbuscular mycorrhiza

Trypan Blue was used for mycorrhizal-structure identification inside the root (Phillips and Hayman 1970). The percentage of root length colonized by hyphae, arbuscules and vesicles was estimated for each sub-sample by a modified line intersection method (McGonigle et al. 1990). A minimum of 50 line-intersections per root sample was scored for AMF structures.

Analysis of dry weight and elemental composition

Shoots and root subsamples were dried 72h at 65°C and dry weights ere estimated. Dried shoots and roots were homogenized and ground at 30 Hz in a mixer mill (MM2224, Retsch, Haan, Germany). Aliquots of 2 mg were weighed for elemental analyses. Nitrogen

and carbon concentrations were determined using an ANCA elemental analyzer/mass spectrometer (Europa Scientific Ltd., Crewe, UK). P concentration of shoots and roots was measured using the molybdate blue method on a Shimadzu UV-160 photospectrometer (Shimadzu Biotech, Duisburg, Germany) after acid digestion (Murphy and Riley 1962).

Identification and characterization of sorghum *Pht1* transporters

Phosphate Pht1 transporters were identified *in silico* using five approaches, based on the full genome sequence of *S. bicolor* (Paterson et al. 2009), available at the Joint Genome Institute (JGI) website (http://genome.jgi-psf.org/Sorbi1/Sorbi1.home.html): (1) known Pht1 protein sequences from *Arabidopsis* and other plants (*e.g., Medicago truncatula, Oryza sativa, Lycopersicon esculentum*, *Populus trichocarpa*) were used to query the *S. bicolor* JGI gene catalog using BLASTP, (2) these plant *Pht1* gene sequences were also used in TBLASTN query against the JAZZ sequence assembly (Paterson et al. 2009), (3) gene models with a predicted phosphate permease domain (IPR004738) were identified using InterProScan and ScanProsite (http://www.expasy.org/tools/scanprosite/), (4) these putative Pht1 transporters were selected on the basis of a Pht1 conserved specific amino acid signature (Karandashov and Bucher 2005), (5) confirmation of these Pht1s models was based on expressed sequence tag (EST) support. Throughout the paper, JGI protein identification (ID) was used to identify these gene models. The final set of Pht1 proteins comprised the following; SbPT1 (JGI Protein ID: 5050188), SbPT2 (JGI Protein ID: 5056998), SbPT3 (JGI Protein ID: 5030542), SbPT4 (JGI Protein ID: 5048813), SbPT5 (JGI Protein ID: 5050189), SbPT6 (JGI Protein ID: 5058886), SbPT7 (JGI Protein ID: 5048812), SbPT8 (JGI Protein ID: 5033142), SbPT9 (JGI Protein ID: 5041365), SbPT10 (JGI Protein ID: 5041362) and SbPT11 (JGI Protein ID: 5036358). Prediction of putative transmembrane (TM) segments for Pht1 proteins was performed using the programs TMHMM, TM pred and Top Pred (http://www.expasy.ch/proteomics). Putative cis-acting elements were searched 2 kb upstream the start codon of the 11 Pht1 genes by DNA-pattern matching analysis.

Isolation and identification of Pht1 transporters of flax

An alignment of amino acid sequences of Pht1 transporters from monocots and dicots were used to identify amino acid regions conserved across this transporter family. Degenerate primers (Forward: dgLuPTf: GAARATGCCNGARCANGC; reverse: dgLuPTr1: TCRAAYGCHACBGTGAACCAG; dgLuPTr2: RNSWYTTNCCYTTNSWYTCNGG) were designed to cover the conserved peptide sequences. *Pht1* gene-specific fragments were amplified by reverse transcriptase polymerase chain reaction (RT-PCR), using degenerate primers and RNA isolated from *G. intraradices* mycorrhizal roots as template. The partial cDNA fragments obtained were extended by the 5'- and 3'-rapid amplification of cDNA ends (RACE) method by using the SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer's recommendations. The RACE products were ligated into T-vectors (pGEM-T Easy Vector, Promega, Madison, WI, USA) and sequenced. Sequences were aligned to each other, and group-specific primers were designed for further RACE reactions to reach the full-length cDNA sequence. These putative Pht1 transporters were inspected for the conserved Pht1 specific amino acid signature (Karandashov and Bucher 2005). Prediction of putative transmembrane segments (TM) for Pht1 proteins was performed using the programs TMHMM, TM pred and Top Pred (http://www.expasy.ch/proteomics).

Phylogenetic analyses

For phylogenetic analysis, the Pht1 amino acid sequences were aligned with ClustalX using the following multiple alignment parameters: gap opening penalty 15, gap extension penalty 0.3, and delay divergent sequences set to 25%; and the Gonnet series was selected as the protein weight matrix. Neighbour joining trees were constructed using the Jones-Taylor-Thornton (JTT) substitution rate matrix for distance computation in MEGA5. Bootstrap analysis was carried out with 500 replicates. Branch lengths (drawn in the horizontal dimension only) are proportional to phylogenetic distances. Gene models used for phylogenetic analysis are given in the supplemental material.

RNA isolation and quantitative RT-PCR analysis

About 100 mg of lyophilized tissue of plants were ground in a ball-mill and total RNA was isolated by using the RNeasy Plant Mini kit (Qiagen, Darmstadt, Germany). The DNA-*free* set (Ambion, Austin, USA) was used to digest DNA after RNA purification. Measurement of *Pht1* transcripts in tissues was performed using a two-step quantitative RT-PCR (qRT-PCR) procedure. Total RNA was quantified with a Nanodrop and then reverse-transcribed (100 ng per reaction) using the iScript cDNA Synthesis kit (Bio-Rad). The resulting cDNA samples were used as templates in real time quantitative PCR reactions with gene-specific primers designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and amplify 3.1 (http://engels.genetics.wisc.edu/amplify; Supplemental Table 3.1). The following criteria were used: product size between 80 and 200 bp, melting temperature 60°C and a GC percentage > 50%. Reactions of qPCR were run using the 7500 real-time PCR system (Applied Biosystems) and using two microliters of 1/10 diluted RT products. The following cycling parameters were applied: 95°C for 3 min and then 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. A control with no cDNA was run for each primer pair. The primer efficiency ranged between 90% and 110%. Target gene expression was normalized (Muller et al. 2002) to the gene encoding ubiquitin in sorghum (JGI Protein ID: 5060159) and in flax (Huis et al. 2010).

3.4 RESULTS

Annotation and distribution of sorghum *Pht1* genes

Fourteen different gene models coding for putative Pht1 transporters were identified in the predicted gene catalog resulting from the automated annotation of *S. bicolor* genome assembly (Version 6.0.176; http://genome.jgi-psf.org/Sorbi1/Sorbi1.home.html). Examination of these gene models revealed two truncated gene models (Protein ID: 5033055 and 5036201), and one gene model (Protein ID: 4801120) missing the Pht1 specific signature (Karandashov and Bucher 2005). The remaining set of eleven putative *Pht1* genes (for *S. bicolor* P_i transporter family 1) were named according to the Commission on Plant Gene Nomenclature as *SORbi;Pht1;1* through *SORbi;Pht1;11*; for simplification, they will be called *SbPT1* through *SbPT11* in this article. Except for SbPT3, ESTs have been identified for these 11 Pht1 transporters, confirming that they are expressed (Supplemental Table 3.2). In the *S. bicolor* genome assembly, these 11 putative *Pht1* genes were located on chromosomes 1, 2, 3, 6 and 7 (Supplemental Figure 3.1). Interestingly *SbPT1* and *SbPT5*, as well as *SbPT4* and *SbPT7*, were present as pairs on chromosome 1, forming a sort of inverted repeats with an intervening putative promoter sequence of only 0.89 kb and 2.77 kb, respectively. However, the sequence similarity within these pairs was not particularly high (73.6 % and 75%, respectively; Supplemental Figure 3.2), indicating that they have evolved independently. *SbPT3, SbPT8, SbPT11* and *SbPT6* are located on chromosome 1, 2, 3 and 7, respectively. *SbPT2, SbPT9* and *SbPT10* were all located on chromosome 6, but none of them appears to be tandem repeat sequences (Supplemental Figure 3.1).

All putative Pht1 proteins of sorghum belonged to the "major facilitator superfamily" (Pao et al. 1998) with 12 predicted TM segments, separated into two blocks of six TM segments by a long hydrophilic loop, and hydrophilic N and C terminus (Supplemental Table 3.2). The Pht1 specific signature (GGDYPLSATIMSE) was conserved and identical for all Pht1 proteins. Eight out of 11 *Pht1* genes are intronless in sorghum (Supplemental Table 3.2). The 2kb up-stream region of the Pht1 genes harbor potential regulatory elements involved in P and AM response, from one in *SbPT5* to 17 in *SbPT4* (Supplemental Figure 3.3). The most prominent element, P1BS (GNATATNC), exhibiting a crucial role in P starvation response, one copy of P1BS is present in the promoter

region of *SbPT1*, *SbPT3*, *SbPT4*, *SbPT6*, *SbPT7* and *SbPT8*, and five copies in the promoter region of *SbPT11*. A root motif box (ATATT or AATAT) is present in the promoter region of all 11 *Pht1* genes. Some of the promoter regions harbor two motifs- OSEROOTNODULE (AAAGAT) and NODCON2GM (CTCTT), which are conserved elements associated with AM and nodule induced leghaemoglobin gene regulation. Additionally, w-boxes (TTGACY) and "unknown elements" (TCTTGTT) were discovered.

Identification of three *Pht1* genes of flax

Three full length sequences of *Pht1* genes were obtained from flax. The three *Pht1* genes were named according to the Commission on Plant Gene Nomenclature as *LINus;Pht1;1* through *LINus;Pht1;3*; for simplification, they will be called *LuPT1* through *LuPT3* in this article. All three Pht1 proteins contained 12 predicted TM domains as described before for sorghum and comprised the Pht1 specific amino acid signature GGDYPLSATIxSE (Karandashov and Bucher 2005), which was slightly modified in LuPT2 to GGDYPLSAVIMSE (Supplemental Table 3.3).

Phylogenetic analysis of the Pht1 phosphate transporters

A neighbor-joining tree of Pht1 phosphate transporters was constructed by multiple sequence alignment, comparing the relevant protein sequences from sorghum and flax and with sequences from other plants and fungi (Figure 3.1). As has been described for other monocotyledon plant species (Nagy et al. 2006), Pht1 proteins of sorghum clustered into four subfamilies (Figure 3.1). SbPT11 is the only transporter belonging to the subfamily I and appears to be closely related to AM-inducible Pht1 transporters ZmPT6 of *Zea mais* (Glassop et al. 2005) and OsPT11 of *Oryza sativa* (Paszkowski et al. 2002). Sorghum also harbors two non-orthologous Pi transporters SbPT9 and SbPT10, belonging to Pht1 subfamily IV, which also contains OsPT13, another AM-inducible P_i transporter of *O. sativa* (Guimil et al. 2005).SbPT9 is closely related to the Pht1 transporters ZmPT5 of *Z. mais* (Nagy et al. 2006). SbPT8 clusters in a small subfamily harboring three other cereal Pht1 transporters of *Brachipodium distachyon* (BdPT3), *Triticum aestivum* (TaPTmyc) and *Hordeum vulgare* (HvPT8);TaPTmyc and HvPT8 are known to be AM-inducible (Glassop et al. 2005). All Pht1 transporters of sorghum except SbPT11 belong to Pht1 subfamilies present only in monocotyledons.

The three Pht1 proteins of flax were distributed in distinct subfamilies (Figure 3.1). LuPT2 clustered in subfamily I harboring AM-inducible Pht1 transporters and was closely related to GmPT7, an AM-inducible P_i transporter of *Glycine max* (Tamura et al. 2012). LuPt1 and LuPT3 both clustered in subfamily III, specific for dicotyledonous plant species (Figure 3.1).

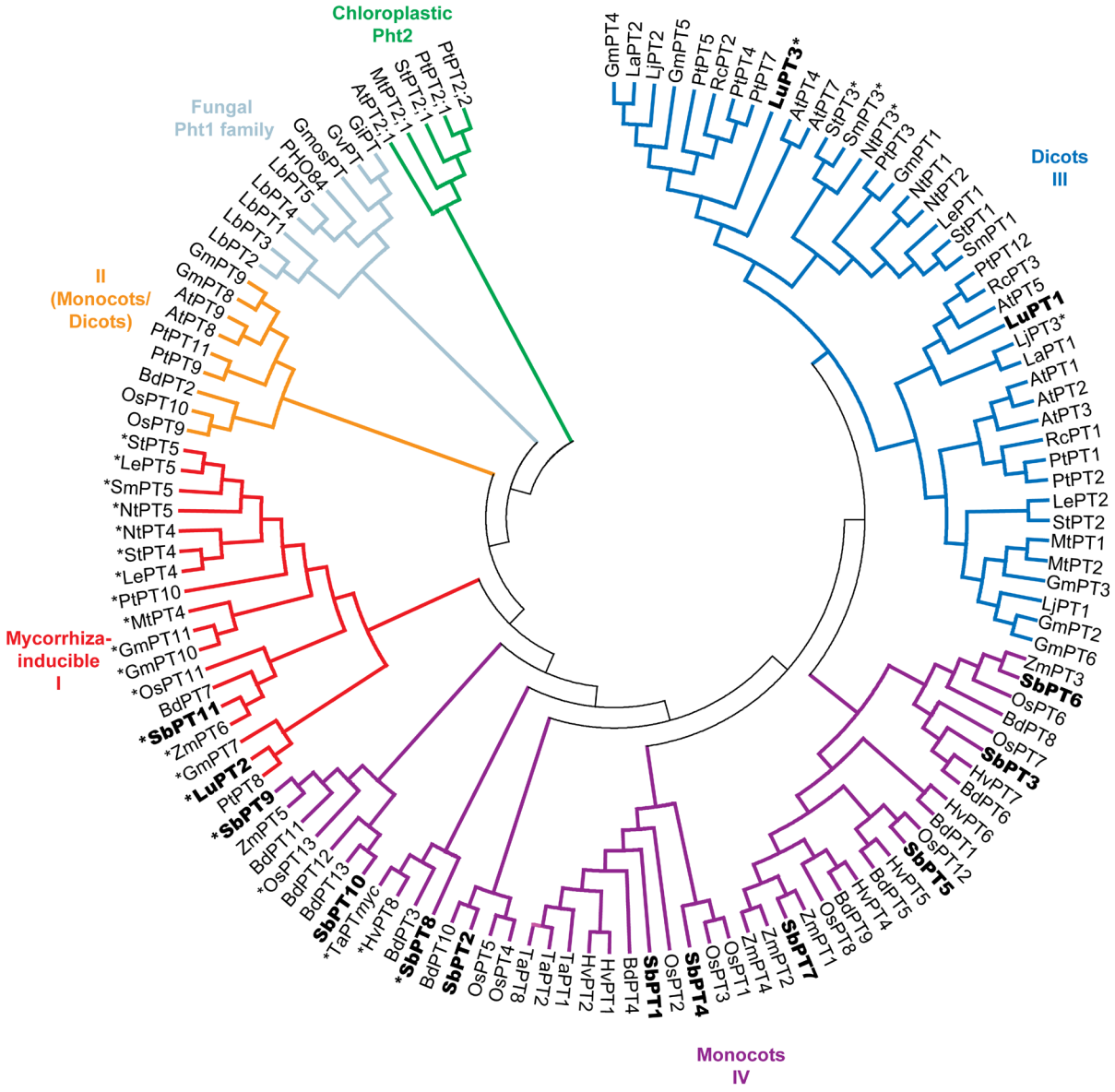

Figure 3.1 Neighbor-joining tree for Pht1 transporters based on aligned sequences (727 amino acids). Sequence names consist of species code (first letter of genus and first letter of species name) and gene name (see Supplemental Material for details). Fungal Pht1 transporters and Chloroplastic Pht2 transporters serve as outgroups. Roman numbers indicate four different plant Pht1 subfamilies thought to different in evolutionary age. The 11 sorghum and the three flax Pht1 transporters are highlighted in bold black. Asterisks indicate Pht1 transporters known to be induced in response to mycorrhiza formation.

Gene expression of Pht1 transporters in different sorghum tissues

Expression of Pht1 transporters in different tissues of sorghum was examined in plants from a grain field. The roots of these plants were colonized by AMF (degree of colonization 42 % ± 3 SEM, data not shown), primarily by *G. mosseae*, as revealed by sequence analysis of fungal ITS sequences (data not shown). In sorghum, ten out of 11 *Pht1* genes were expressed in roots; only *SbPt3* was not detectable in any of tissues investigated (Figure 3.2). Highest expression levels were measured for *SbPT7* in all tissues and for *SbPT11* in roots. *SbPT2, SbPT6* and *SbPT7* were constitutively expressed in all the tissues. *SbPT1* and *SbPT10* were only detectable in mycorrhizal roots. *SbPT4* was only expressed in vegetative tissues. *SbPT9* was detected in mycorrhizal roots and leaves. Besides in mycorrhizal roots, *SbPT11* was expressed in both female and male inflorescence, whilst *SbPT5* and *SbPT8* were only expressed in stamina.

Influence of P_i availability and arbuscular mycorrhizae on *Pht1* gene expression in sorghum roots

The expression level of *Pht1* genes studied in sorghum roots under conditions of low P_i supply, either in the absence of AMF or in the presence of *G. intraradices* or *G. mosseae* (Figure 3.3). Expression of *SbPT8, SbPT9, SbPT10 and SbPT11* genes was significantly enhanced in mycorrhizal roots. The expression of *SbPT9* was significantly enhanced in roots colonized with *G. mosseae* only (Supplemental Figure 3.5). *SbPT11* was only detectable in mycorrhizal roots. In contrast, expression of *SbPT1, SbPT2* and *SbPT6* was significantly repressed in mycorrhizal roots. *SbPT4, SbPT5* and *SbPT7* transcripts were not modified upon AM colonization.

Additional P_i fertilizer only weakly affected sorghum plant growth performance, but P content of plants was significantly higher with additional P_i supply (Supplemental Figure 3.4). All mycorrhizal plants exhibit an arbuscular colonization of 46 % ± 3 SEM without significant differences among P treatment and AMF species (p-value = 0.91). Plants growing in P_i-poor conditions were compared with plants growing with additional P_i fertilization in non-mycorrhizal roots and in roots colonized by *G. intraradices* or *G. mosseae*. Genes exhibiting a modification in expression of at least the factor two are considered to be up- or down-regulated. In non-mycorrhizal roots, P_i supply significantly

repressed the expression of *SbPT1* and *SbPT8*, but significantly enhanced *SbPT6* (Supplemental Figure 3.5). In contrast, in mycorrhizal roots, neither *SbPT1* nor *SbPT8* were down-regulated upon additional P_i fertilization. *SbPT6* was also significantly overexpressed in mycorrhizal roots (Supplemental Figure 3.5). Expression of the *SbPT2, SbPT4, SbPT5, SbPT7, SbPT9, SbPT10* and *SbPT11* was not or marginally modified by P_i availability. Remarkably, expression of *SbPT5* was repressed with additional P_i supply in non-mycorrhizal roots and roots colonized by *G. intraradices*, but rather enhanced in roots colonized with *G. mosseae*.

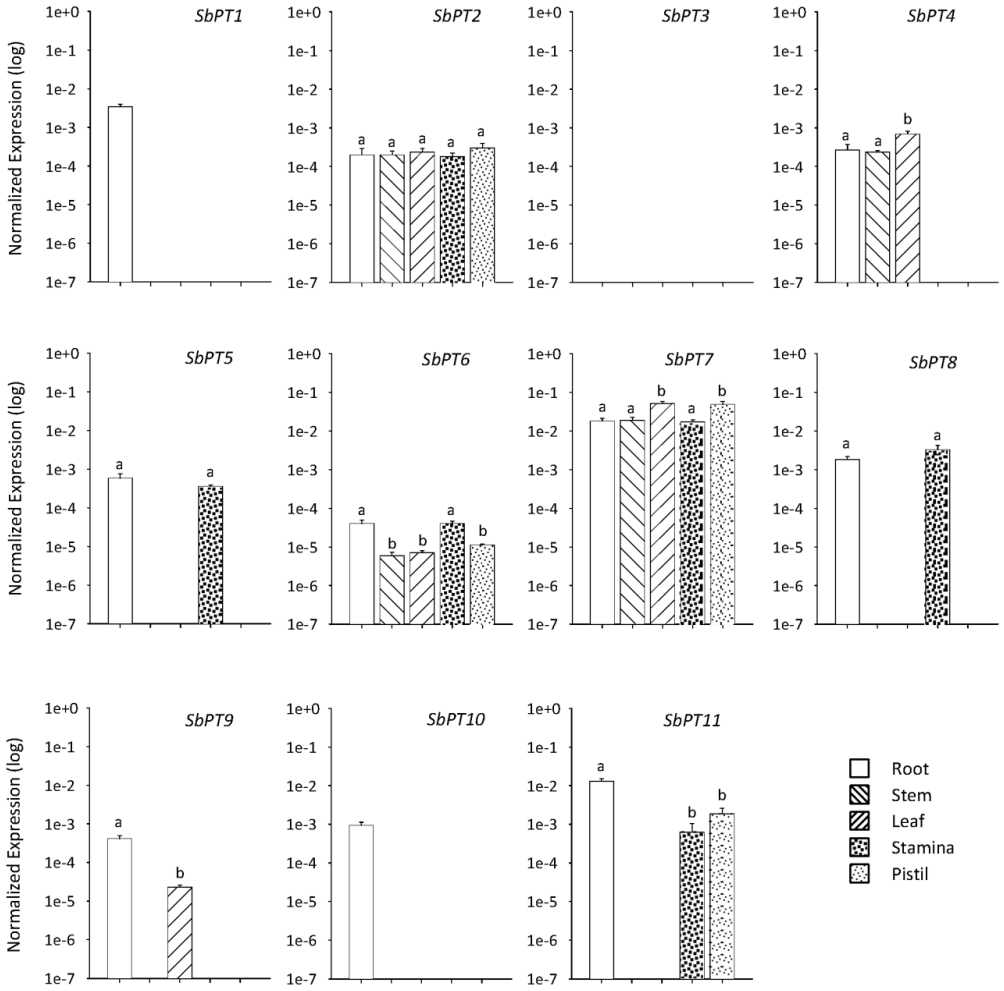

Figure 3.2 Quantification by qRT-PCR analysis of transcript levels of the 11 sorghum *Pht1* genes in different tissue types. Sorghum plants were harvested in a grain field and roots were colonized by *Glomus mosseae* isolates (42 % ± 3). Transcript levels were normalized against ubiquitin. Values are means of three replicates, each replicate represented at least a pool of three plants. Error bars represent SEM. For each gene, different lowercase letters above bars indicate a significant difference ($P \leq 0.05$) among treatments, according to LSD test.

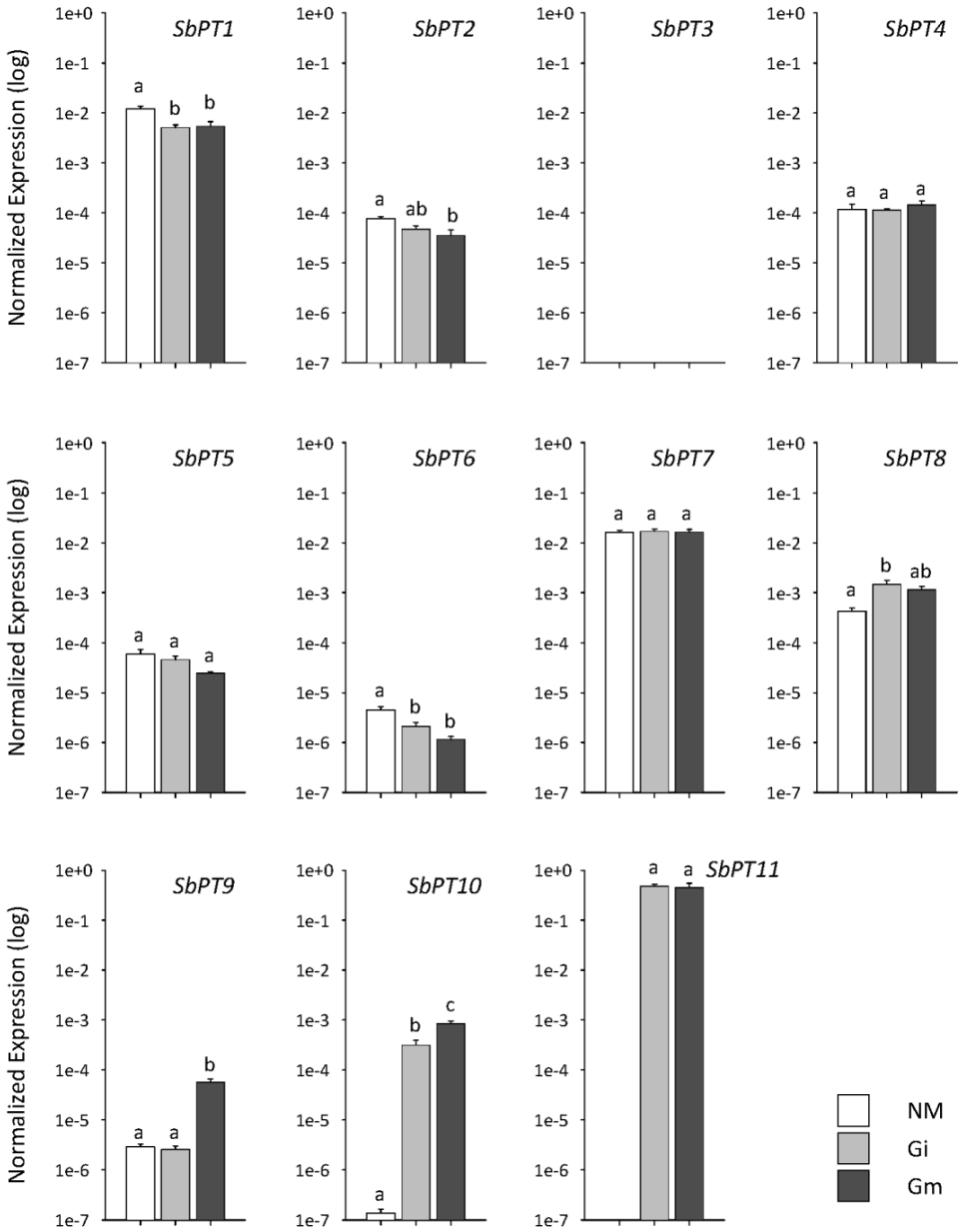

Figure 3.3 Quantification by qRT-PCR of the transcript levels of the 11 sorghum *Pht1* genes in non-mycorrhizal roots (NM) and roots colonized by *G. intraradices* (Gi) or *G. mosseae* (Gm). Transcript levels were normalized against ubiquitin. Values are means of four replicates, error bars represent SEM. For each gene, different lowercase letters above bars indicate a significant difference (P≤0.05) among treatments, according to LSD test.

Pht 1 gene expression in sorghum and flax sharing a mycorrhizal network

As shown in Figure 3.4, *Pht1* genes of sorghum exhibited similar expression patterns upon mycorrhization in monoculture as in single pot culture described above (Figure 3.3). Again, *SbPT8*, *SbPT9*, *SbPT10* and *SbPT11* were strongly induced in association with AMF; moreover, *SbPT10* and *SbPT11* were even only detected in mycorrhizal roots (Figure 3.4). The expression of *SbPT1* was repressed by the factor 100 in AM symbiosis. *SbPT2* and *SbPT5* were significantly upregulated in association with *G. intraradices,* which contrasts to the repression measured in single pots (Figure 3.3). *SbPT6* was significantly repressed in roots colonized by *G. mosseae* and in roots colonized by *G. intraradices* in mixed cultures. Similarly, expression of *SbPT10* was more enhanced when colonized by *G. mosseae* than by *G. intraradices* (Figure 3.4). However, different culture systems modified the expression of *Pht1* genes in sorghum only marginally compared to the effect of AMF colonization. In normalized expression, only the expression level of *SbPT6* was significantly reduced in mixed culture of compare to monoculture when associated with *G. intraradices*, and the expression level of *SbPT10* was significantly increased in a CMN with flags formed by *G. mosseae* (Figure 3.4).

The expression of the three *Pht1* genes of flax was significantly modified by AM symbiosis in monoculture. Expression of *LuPT1* was repressed in mycorrhizal roots, whilst the expression level *LuPT2* and *LuPT3* showed increased expression levels (Figure 3.5). In flax, different culture systems had an impact on the expression of *LuPT2* and *LuPT3*; in association with *G. mosseae, LuPT2 and LuPT3* were significantly less expressed in mixed-culture than in monoculture and were showing similar expression levels as in non-mycorrhizal roots (Figure 3.5).

The differences in expression of AM-inducible *Pht1* genes between mono- and mixed culture are more precisely displayed in comparing their relative expression (Table 3.1). In sorghum roots associated with *G.mosseae*, *SbPT9* and *SbPT10* were more expressed in mixed culture than in monoculture, whereas in association with *G. intraradices* no difference was observed. In contrast, the AM-induced *Pht1* genes of flax, *SbPT2* and *SbPT3*, were repressed in mixed culture when associated with *G. mosseae*, but tended to be up-regulated when associated with *G. intraradices*.

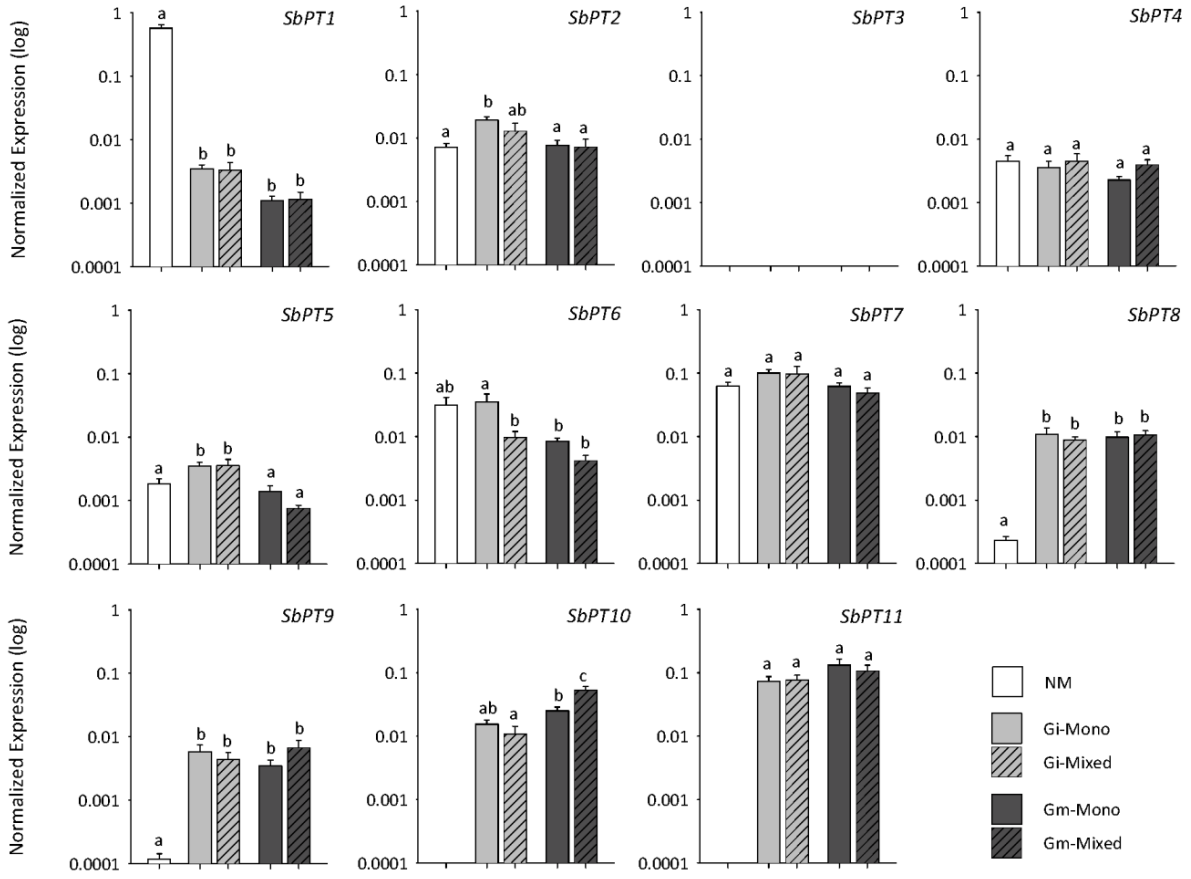

Figure 3.4 Quantification by qRT-PCR analysis of transcript levels of the 11 sorghum *Pht1* genes in non-mycorrhizal roots (NM) or in roots connected to a mycorrhizal network built by G*lomus. intraradices* (Gi) or *G. mosseae* (Gm) shared with a co-cultured sorghum (Mono) or with a co-cultured flax plant (Mixed). Transcript levels were normalized against ubiquitin. Values are means of four replicates, error bars represent SEM. Different lowercase letters above bars indicate a significant difference (P≤0.05) among treatments, according to LSD test.

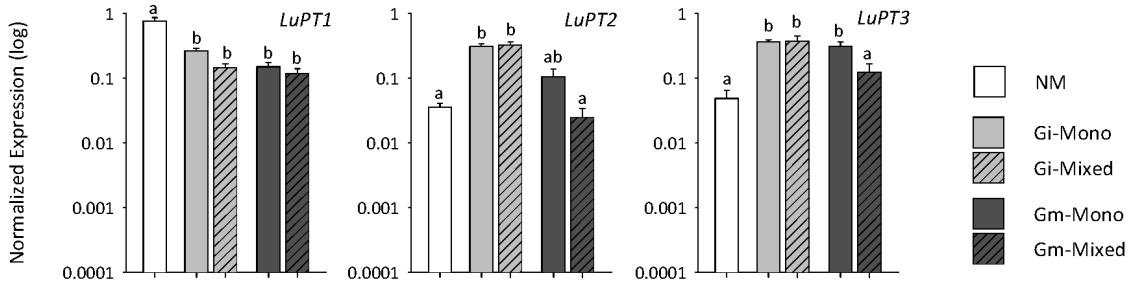

Figure 3.5 Quantification by qRT-PCR analysis of transcript levels of the 3 flax *Pht1* genes in non-mycorrhizal roots (NM) or in roots connected to a mycorrhizal network build by *Glomus intraradices* (Gi) or *G. mosseae* (Gm) shared with a co-cultured flax (Mono) or with a co-cultured sorhum plant (Mixed). Transcript levels were normalized against ubiquitin. Values are means of four replicates, error bars represent SEM. Different lowercase letters above bars indicate a significant difference (P≤0.05) among treatments, according to LSD test.

3.5 DISCUSSION

Phylogenetic relation of Pht1 transporters

The phylogenetic analysis revealed the relation of the 11 sorghum transporters (Figure 3.1) with members of previously described Pht1 subfamilies (Karandashov and Bucher 2005). Subfamily I harbors the AM-inducible P_i transporters from both monocotyledonous and dicotyledonous plant species, indicating their evolution before the separation of the two plant groups. In sorghum SbPT11, the protein encoded by one of the AM-induced *Pht1* genes, fall into this group. The three other AM-inducible P_i transporter of sorghum, SbPT8, SbPT9 and SbPT10, fall into divergent subfamilies harboring transporters of monocotyledonous plant species, which are partially AM-inducible. These subfamilies seems to be evolutionary younger than the subfamily I, because they comprise proteins solely from monocotyledonous species. This indicates a separate evolutionary gain of AM-inducibility of genes involved in P uptake, both in monocotyledonous and dicotyledonous species (Karandashov et al. 2004, Nagy et al. 2006). In addition, the Pht1 transporters SbPT1 to SbPT7 fall also in the monocot-specific subfamily IV. Genes coding for SbPT1 to SbPT8 have no introns, suggesting that they arise from duplication events from a primordial gene (Lynch and Conery 2000). The tandem repeat sequences of *SbPT1*/*SbPT5* and *SbPT4*/*SbPT7* are likely to be most recent duplications.

Expression of *Pht1* genes in different sorghum tissues

Pht1 genes exhibit substantial differences in their expression in the various organs examined. *SbPT2, SbPT6* and *SbPT7* were constitutively expressed in sorghum and thus may be involved in P homeostasis (Jia et al. 2011). The transcripts coding for *SbPT5*, *SbPT8* and *SbPT11* were expressed in roots, but in addition also strongly expressed in inflorescence, mainly in stamina, similar to their homolog from *Arabidopsis* and maize, which were suggested to fulfill P_i uptake in the elongation pollen tube (Mudge et al. 2002, Nagy et al. 2006). The specific expression of *SbPT9* in leaves in addition to roots indicates a role in P remobilization in leaves, as suggested for a homologous transporter in poplar (Loth-Pereda et al. 2011). *SbPT1* was only detected in the roots and down-regulated

upon P fertilization in non-mycorrhizal roots (Figures 3.2 and 3.3), indicating a role in direct P_i uptake by the roots (Liu et al. 1998b, Karandashov and Bucher 2005, Ai et al. 2009). With respect to root expression, most interestingly, *SbPT10* and *SbPT11* were detected in AMF-colonized (Figure 3.3), and therefore probably involved in symbiotic P_i uptake (Smith et al. 2011). *SbPT3* was not detected in any of the examined tissue types under the present experimental conditions (Figure 3.1, 3.2, 3.4 and Supplemental Figure 33.). Thus, this *Pht1* gene could be expressed under specific conditions, which were not included in our experimental set up, or, alternatively, the expression levels of *SbPT3* were too low to be detected.

Physiological measurements have shown that P starved plants rapidly increase their capacity in phosphate uptake by transcriptional up-regulation of *Pht1* genes (Smith et al. 2003a). One of the best-characterized regulatory element involved in P regulation is the transcription factor PHR1, which activates a subset of P starvation induced genes by binding on cis-acting P1BS elements (Rubio et al. 2001, Nilsson et al. 2007). In the promoter region of seven of the 11 *Pht1* genes of sorghum, we found at least one P1BS element (Supplemental Figure 3.4), including *SbPT1, SbPT6* and *SbPT8*. Interestingly, *SbPT1* and *SbPT8* were strongly downregulated in nonmycorrhizal roots in response to P fertilization, while *SbPT6* was clearly upregulated under the same conditions (Supplemental Figure 3.5), indicating that similar regulatory elements may have opposite functions in gene expression.

In the roots, two genes were practically exclusively expressed in the presence of mycorrhiza (Figure 3.3), namely *SbPT11*, which is also expressed in flower organs, which belongs to subfamily I of Pht1 genes, comprising mycorrhiza-inducible Pht1 genes of both in monocots and dicots, and *SbPT10*, which belongs to the monocot-specific subfamily IV. Some of the members of the latter group have also been shown to be mycorrhiza inducible (Figure 3.1). Several plant species, e.g. *O. sativa, L. esculentum, S. tuberosum* possess multiple AM-induced *Pht1* genes (Guimil et al. 2005, Nagy et al. 2005), resulting in a functional redundancy of symbiotic P_i transport (Nagy et al. 2005), whereas other plant species harbor a single AM-induced *Pht1* gene which appears to be crucial for the symbiotic P acquisition (Javot et al. 2007a).

AM-dependent induction of P_i transporters is often accompanied by the down-regulation of other *Pht1* genes; in particular those suggested being involved in the direct P uptake

(Rausch et al. 2001, Paszkowski et al. 2002, Glassop et al. 2005). Such AM-dependent down-regulation was apparent in the low transcript level of *SbPT1* in mycorrhizal roots, earlier suggested being involved in direct P uptake. Transcriptional AM-inducibilty has been suggested to be regulated by two cis-acting regulatory elements, P1BS and MYCS, in solanaceous species (Chen et al. 2011); P1BS, in particular, might have a double role as a regulatory element in response to P_i availability as well as to AM symbiosis (Smith et al. 2011). However, P1BS is only present in the AM-inducible *Pht1* genes *SbPT8* and *SbPT11*, whilst *SbPT10* does not harbor a P1BS element in their promoter region (Supplemental Figure 3.4). The transcriptional regulation of AM-inducible genes is still poorly understood; the discovery of more AM-specific regulatory elements, active in broad range of mycorrhizal plants will shed new light on AM-inducibility.

Table 3.1 Relative expression of AM-induced *Pht1* genes of sorghum and flax

AMF species	Plant species			
	Sorghum		Flax	
	Pht1 gene	Rel. Expression	*Pht1* gene	Rel. Expression
G. intraradices	*SbPT1*	1.18 ± 0.40	*LuPT1*	0.57 ± 0.16
	SbPT8	1.08 ± 0.16	*LuPT2*	1.60 ± 0.28
	SbPT9	1.09 ± 0.34	*LuPT3*	1.54 ± 0.58
	SbPT10	0.98 ± 0.47		
	SbPT11	1.43 ± 0.41		
G. mosseae	*SbPT1*	1.23 ± 0.41	*LuPT1*	0.96 ± 0.19
	SbPT8	1.33 ± 0.24	*LuPT2*	0.31 ± 0.11
	SbPT9	4.27 ± 1.32	*LuPT3*	0.46 ± 0.16
	SbPT10	2.99 ± 0.39		
	SbPT11	1.00 ± 0.26		

Quantification by RT-qPCR analysis of the transcript levels of AM-induced *Pht1* genes in sorghum and flax roots as affected by mono- and mixed culture. Roots were colonized by G*lomus intraradices* and *G. mosseae*. Genes were considered to be biologically regulated, if modified by more than the factor two. Transcript levels in roots grown in monoculture were used as control values for relative expression. Transcripts were normalized against ubiquitin. Values are means (± SEM) of four replicates. CMN-mediated P_i uptake was estimated by measuring ^{33}P uptake only accessible for AMF hyphae. In comparison of mono- versus mixed culture, sorghum received 0.1 and 0.6 times less CMN-mediated P_i in association to *G. intraradices* and to *G. mosseae,* respectively. In contrast, flax received 2.1 and 1.8 times more CMN-mediated P_i in association to *G. intraradices* and to *G. mosseae,* respectively.

Role of transcriptional regulation of Pht1 transporters in P_i acquisition *via* a CMN

In monoculture, two individual sorghum plants received an equal share of CMN-mediated P_i, indicating a similar ability to acquire P_i *via* the CMN (Walder et al. 2012). Nevertheless, sorghum was acquiring the double amount of P *via* the CMN built by *G. mosseae* compared to *G. intraradices* (no significant differences were found in total and arbuscular colonization levels, neither between fungal nor between plant species). This better functional compatibility in association with *G. mosseae* was underlined by the stronger induction of the AM-inducible *Pht1* genes (Figures 3.3 and 3.4S) and by exclusive root colonization in field. Indeed, AM associations revealed preferences under field conditions (Helgason et al. 2002, Davison et al. 2011). Our experiment was reflecting the interdependence of preference and compatibility in the sorghum - *G. mosseae* association.

In mixed culture sharing the mycorrhizal network with associated flax, sorghum acquired 6% of P_i *via* the CMN built by *G. intraradices* and 52% when built by *G. mosseae* (Walder et al. 2012). Surprisingly, expression of the two main AM-induced *Pht1* genes of sorghum, *SbPT10* and *SbPT11*, was very strongly induced even in the presence of *G. intraradices*, under conditions where sorghum received virtually no CMN-mediated P_i, and thus exhibited an inactive mycorrhizal pathway (Figures 3 and 4). It is worth noting, however, that the expression of *SbPT9* and *SbPT10* was enhanced in mixed culture with flax when the CMN was formed by *G. mosseae,* i.e. under conditions where sorghum obtained more P_i through the mycorrhizal pathway (Table 3.1). In mixed-cultures, flax acquired the double amount of P_i compared to the monoculture *via* both CMNs (Walder et al. 2012), Nevertheless, the AM-induced *Pht1* genes were less expressed in mixed cultures compared to monocultures when the CMN was formed by *G. mosseae*, and dropped down even to the non-mycorrhizal level (Figure 3.5 and Table 3.1).

Our data show, in accordance with current literature (Smith and Smith 2011), that transcriptional regulation of specific members of the Pht1 transporter family occurs, both in sorghum and in flax, in response to mycorrhiza formation. However, in the situation of mixed cultures between sorghum and flax engaged in a CMN, transcriptional regulation of these transporters is not related to the differential gain of P by the interconnected plants. Besides the expression level of the transporter genes, post-transcriptional regulation and the rates of protein turnover influence the number of

transporter in the plasma membrane (Smith and Smith 2011). Transcriptional regulation appears to be crucial for the induction of Pht1 transporters during the initialization of the symbiotic P exchange at periarbuscular membrane (Harrison et al. 2002), whereas fine-tuned regulation of the transport system probably may be more dependent on posttranscriptional regulation and protein turnover.

Even if the expression of AM-inducible *Pht1* genes of sorghum and flax were only marginally related to P_i supply via mycorrhizal network, the relative expression elucidated differences between the culture systems (Table 3.1). In mixed culture and *G. mosseae* as fungal partner, *SbPT9* and *SbPT10* were up-regulated, and *LuPT2* and *LuPT3* were repressed. In mixed culture and *G. intraradices* as fungal partner, flax genes were only slightly up-regulated. Therefore, it appears that expression of AM-inducible *Pht1* genes is affected not only by the fungal partner, but also by the interconnected plant species. To explain these data, jumping in applied ecology could help. A genotype has a traditional phenotype exhibited in association with individuals of the same species. However, in interacting with individuals of other species, the traditional phenotype is extended to another level, called community or ecosystem phenotype (Whitham et al. 2006). In other words, here, flax and sorghum plants interconnected *via* the CMN are forming a unique "community phenotype" (Whitham et al. 2006), in which expression of the AM-inducible *Pht1* genes could be mediated by complex interactions between the three partners (sorghum, flax, AMF species).

Acknowledgments

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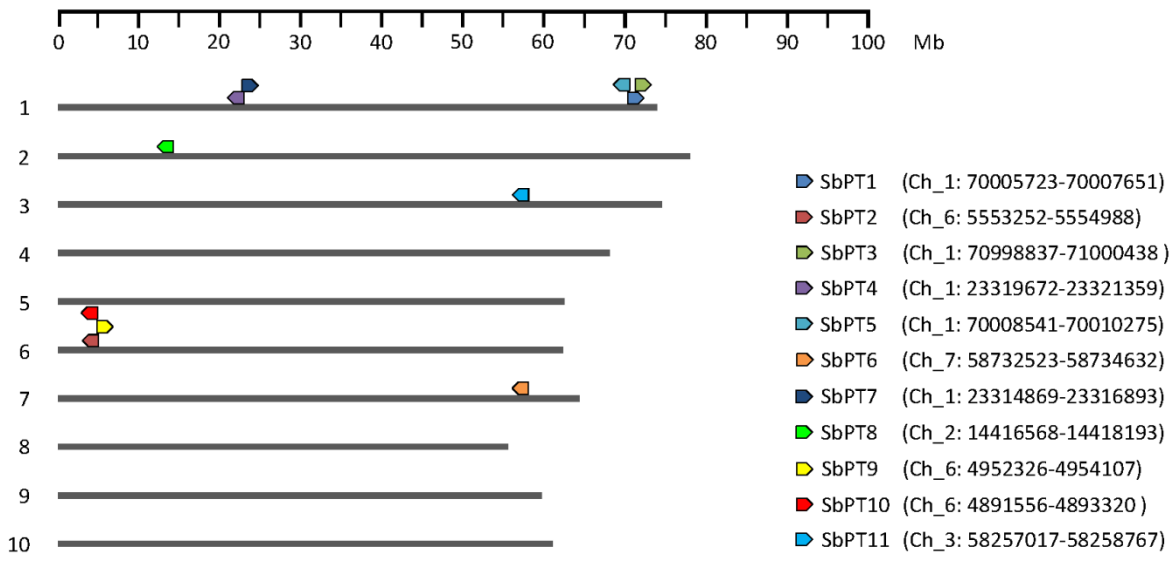
3.6 SUPPLEMENTAL MATERIAL

Sequences used for phylogenetic analysis

The phylogenetic analysis presented in Figure 1 is based on the putative amino acid sequences of phosphate transporters based on DNA sequences, as archived in the UniProt data base. Sequences retrieved from NCBI GenBank were:

Arabidopsis thaliana; AtPT1 (Q8VYM2), AtPT2 (Q96243), AtPT3 (O48639), AtPT4 (Q96303), AtPT5 (Q8GYF4), AtPT6 (Q9ZWT3), AtPT8 (AEE79268.1), AtPT9 (Q9S735); *Medicago truncatula*: MtPT1 (AAB81346), MtPT2 (AAB81347), MtPT4 (AAM76743), MtPT2;1 (XP_003628943); *Glycine max*: GmPT1 (ACP19339), GmPT2 (ACN80147), GmPT3 (ACP19343), GmPT4 (ACP19342), GmPT5 (ACP19340), GmPT6 (ACP19341), GmPT7 (ACP19345), GmPT8 (ACP19338), GmPT9 (ACP19347), GmPT10 (ACP19346), GmPT11 (ACP19344); *Lotus japonicus*: LjPT1 (BAE93354), LjPT2 (BAE93355), LjPT3 (BAE93353); *Lupinus albus*: LaPT1 (AAK01938), LaPT2 (AAK38197); *Lycopersicon esculentum*: LePT1 (AAB82146), LePT2 (AAB82147), LePT3 (AAV97729), LePT4 (AAX85193); *Ricinus communis*: RcPT1 (XM_002531486), RcPT2 (XM_002524576), RcPT3 (XM_002517998); *Lycopersicon esculentum*: LePT1 (AAB82146), LePT2 (NP_001234043), LePT4 (NP_001234674), LePT5 (AAX85194); *Solanum tuberosum*: StPT1 (CAA67395), StPT2 (CAA67396), StPT3 (AAV97729), StPT4 (AAW51149), StPT5 (AY885654), StPT2;1 (AAT35816); *Solanum melongena*: SmPT1 (ABK63959), SmPT3 (ABK63963), SmPT5 (ABK63969) *Nicotiana tabacum*: NtPT1 (AAF74025), NtPT2 (BAB21545), NtPT3 (ABK63964), NtPT4 (ABK63967), NtPT5 (ABK63970); *Populus trichocarpa*: PtPT1 (XP_002315705), PtPT2 (XP_002332753), PtPT3 (XP_002332751), PtPT4 (XP_002306844), PtPT5 (XP_002302047), PtPT6 (XP_002306623), PtPT7 (XP_002306845), PtPT8 (XP_002329198), PtPT9 (XP_002300626), PtPT10 (XP_002331845), PtPT11 (XP_002307816), PtPT12 (XP_002300153), PtPT2;1 (XP_002314598), PtPT2;2 (XP_002311765); *Oryza satisa*: OsPT1 (AAN39042), OsPT2 (AAN39043), OsPT4 (AAN39045), OsPT5 (AAN39046), OsPT6 (AAN39047), OsPT7 (AAN39048), OsPT8 (AAN39049), OsPT9 (AAN39050), OsPT10 (AAN39051), OsPT11 (AAN39052), OsPT12 (AAN39053), OsPT13 (AAN39054); *Hordeum vulgare*: HvPT1 (AAN37900), HvPT2 (AY187020), HvPT4 (AY187025), HvPT5 (AAO72435), HvPT6 (AAN37901), HvPT7 (AAO72436), HvPT8 (AAO72440); *Triticum aestivum*: TaPT1 (CAC69857), TaPT2 (CAC69855), TaPT8 (AAP49822), TaPT*myc* (AH25730); *Zea mays*: ZmPT1 (NP_001105269), ZmPT2 (NP_001105816), ZmPT3 (AAY42387), ZmPT4 (AAY42388), ZmPT5 (AAY42389), ZmPT6 (NP_001105776); *Brachypodium distachyon*: BdPT1 (XP_003558115), BdPT2 (XP_003560773), BdPT3 (XP_003557302), BdPT4 (XP_003558800), BdPT5 (XP_003562075), BdPT6 (XP_003573982), BdPT7 (XP_003569484), BdPT8 (XP_003573220), BdPT9 (XP_003573982), BdPT10 (XP_003581012), BdPT11 (XP_003581010), BdPT12 (XP_003581013), BdPT13 (XP_003581014); *Laccaria bicolor*: LbPT1 (XP_001889013), LbPT2 (XP_001889026), LbPT3 (XP_001889028), LbPT4 (XP_001880970), LbPT5 (XP_001888254); *Glomus intraradices*: GiPT (AAL37552); *Glomus mosseae*: GmosPT (AAZ22389); *Glomus versiforme*: GvPT (AAC49132); *Saccharomyces cerevisiae* S88c: ScPHO84 (NP_013583).

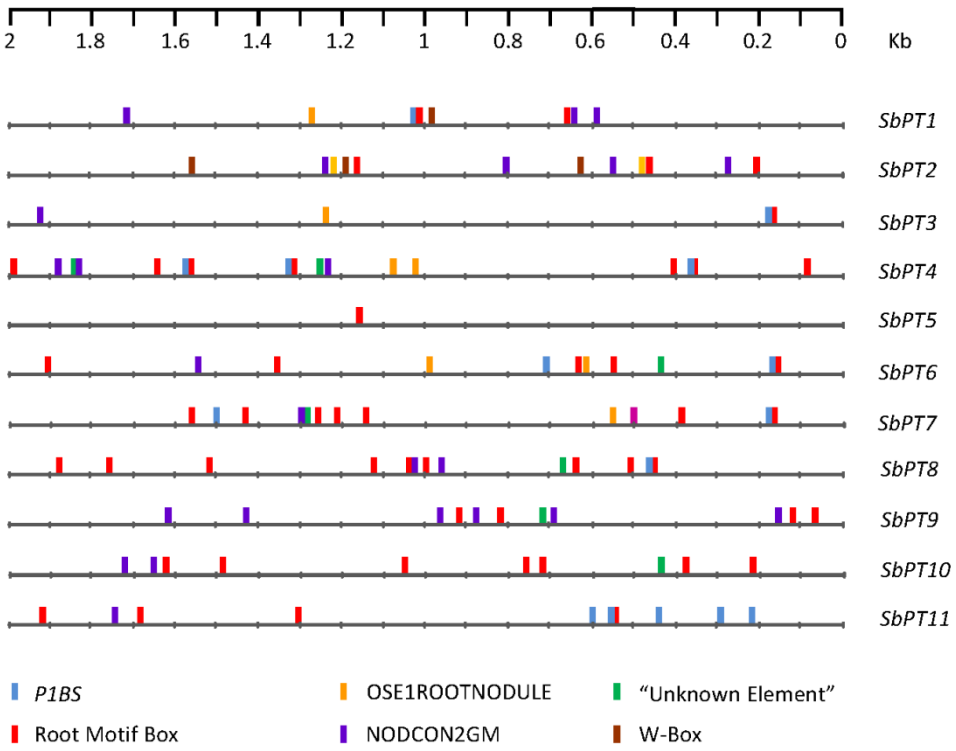
Supplemental Figures



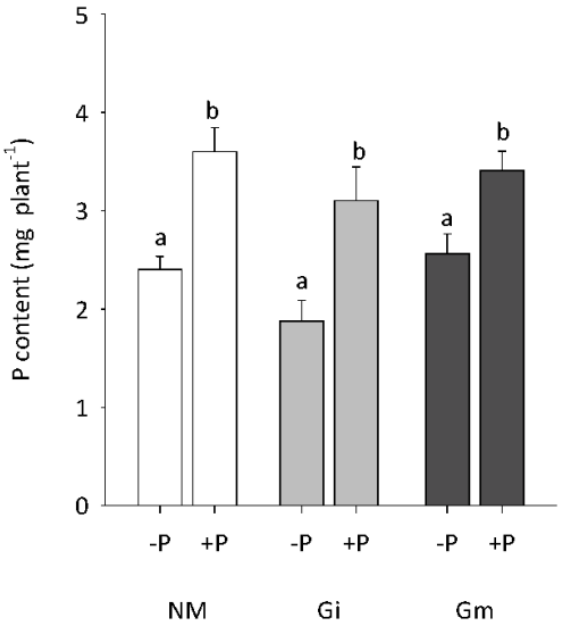
Supplemental Figure 3.1 Gene distribution of the 11 *Pht1* genes on the 10 chromosomes (Ch) of *Sorghum bicolor* according to (Paterson et al. 2009).

	% of Identity										
	SbPT1	SbPT2	SbPT3	SbPT4	SbPT5	SbPT6	SbPT7	SbPT8	SbPT9	SbPT10	SbPT11
SbPT1		70.2	68.2	83.6	73.6	72.7	73.9	57.7	53.7	53.1	56.4
SbPT2	79.2		68.4	71.8	71.6	73.9	73.7	61.5	54.6	53.3	52.9
SbPT3	82	80.7		68.5	68.4	74.8	69.1	56.8	51.7	50.9	51.5
SbPT4	91.4	83.1	82.7		75	73.4	75	59.4	54.6	53.3	57
SbPT5	83.6	83.4	81.5	85		74.5	80.7	60	55	52.5	55.7
SbPT6	82.6	84.3	86.3	84.1	84.3		78.4	61.2	55.6	53.1	55.7
SbPT7	84.1	86	82.8	86.3	88.4	87.8		60.5	55.9	54.3	58.2
SbPT8	73.8	75.8	74.5	75.2	76.5	77.1	77.6		49	47.5	49.4
SbPT9	70.5	69.8	69.2	71.1	69.9	70.4	70.6	64.9		55.6	46.7
SbPT10	69.5	71.8	69.2	69.5	69	70.5	71.2	67.3	71.7		47.5
SbPT11	70.9	70.4	69.5	70.4	70.9	71.1	73.3	67.9	62.3	66.4	
	% of Similarity										

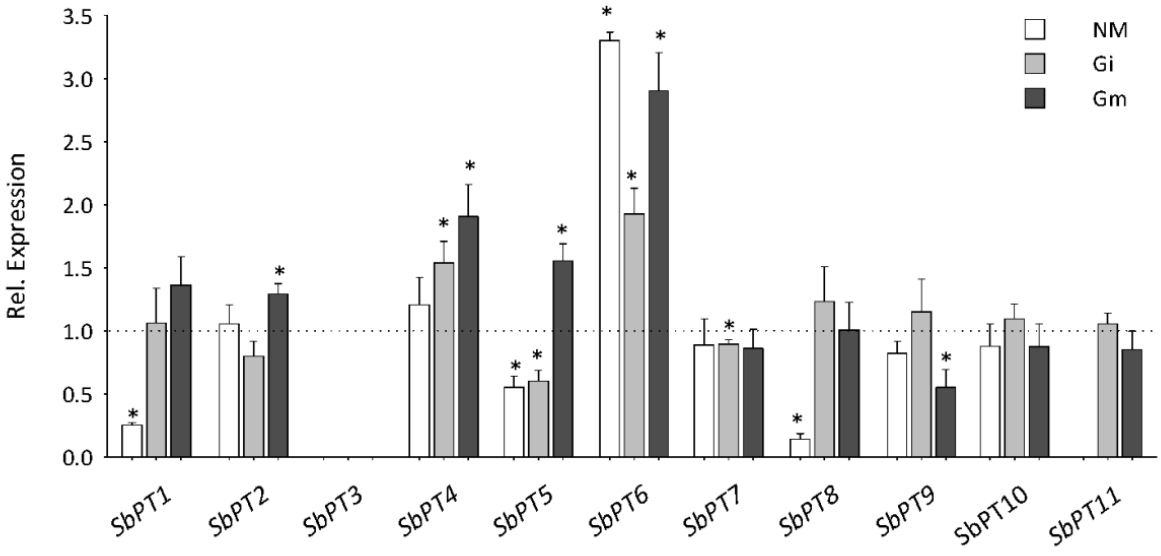
Supplemental Figure 3.2 Similarity/identity matrix of *Sorghum bicolor* Pht1 protein sequences.



Supplemental Figure 3.3 Putative cis-regulatory elements involved in P_i and AM response in the promoter region of the 11 *Pht1* genes. The putative cis-regulatory elements were screened with DNA-pattern matching analysis within 2 kb upstream region of the start codon. P1BS, GNATATNC; root motif box, ATATT or AATAT; OSE1ROOTNODULE, AAAGAT; NODCON2GM, CTCTT; W-box, TTGACY and an unknown element, TCTTGTT.



Supplemental Figure S3.4 Phophorus (P) content of plants as affected by P availability (low P availability (-P), high P availability by addition of P fertilizer(+P)) in non-mycorrhizal roots (NM) and roots colonized by *Glomus intraradices* (Gi) or *G. mosseae* (Gm). Values are means of four replicates, error bars represent SEM. Different lowercase letters above bars indicate a significant difference ($P \leq 0.05$) among treatments, according to LSD test.



Supplemental Figure 3.5 Quantification by RT-qPCR analysis of the transcript levels of the 11 sorghum *Pht1* genes in sorghum roots in response to P_i availability. Roots were non-mycorrhizal (NM), or colonized by G*lomus. intraradices* (Gi) and *G. mosseae* (Gm). Genes were considered to be biologically regulated if affected by more than the factor two. Transcript levels in roots without any P_i fertilization were used as control values for relative expression. Transcripts were normalized against ubiquitin. Values are means of four replicates, error bars represent SEM. Asterisk above bars indicate a significant difference ($P \leq 0.05$) according to Student's t-test.

Supplemental Tables

Supplemental Table S3.1 Primers for qRT PCR analysis of Pht1 transcripts of sorghum and flax.

Name	Sequence (5'-3')	JGI Protein ID
SbPT1a	GGCCAAGGTGCTCAAGAAG	5050188
SbPT1b	GGAGGAACTGCACCGAGAAG	
SbPT2a	ACTAAGCAGCAGCCTCCGTA	5056998
SbPT2b	AAGCCACAAGGAAACCATTG	
SbPT3a	TACTCGCGTATGAACATGCC	5030542
SbPT3b	TCCTCCTTATTGCCGATGTC	
SbPT4a	GGCGCCGTCGTACCAGGACAA	5048813
SbPT4b	GAGCGCCGCCGGGATGGT	
SbPT5a	GAGAATCTGGACGAGATCAC	5050189
SbPT5b	CAGGTTCTGGCTGTAGTAGG	
SbPT6a	CAAGCTCGGCCGTAAGAAGG	5058886
SbPT6b	GCCAGAAGCGGAAGAAGCAC	
SbPT7a	GGACACCAGCAAGGACAAC	5048812
SbPT7b	CGCGATGGAGCAGATGAC	
SbPT8a	GCAGCGAGGCCAATGAGACT	5033142
SbPT8b	TTGGCTCCGGTAGGAAGCAG	
SbPT9a	GAGGACGAGCCGTTCAAGAG	5041365
SbPT9b	CGCGACGGAGAAGAAGTACC	
SbPT10a	CACCATGTGCTGGTTACTTC	5041362
SbPT10b	GATAATCGCCTGAGTACGTG	
SbPT11a	CGTGGTTCCTTCTGGACATA	5036358
SbPT11b	TCTCGAACACCTCCTTGAGT	
SbUBIa	CAAGGAGTGCCCCAACAC	5060159
SbUBIb	TGGTAGGCGGGTAAAGCAAA	
LuPT1a	GGGAATGAAGCAGCTAGCAC	-
LuPT1b	TTCCTTCTACCCATGCCATC	
LuPT2a	GATTCGAACGGGATCAAGAA	-
LuPT2b	GACGGCAACCACTTTCTCAT	
LuPT3a	AGCAGCAACACAACAGCTTG	-
LuPT3b	CGTCGTGACTGGAACAGCTA	
LuUBIa	CTCCGTGGAGGTATGCAGAT*	
LuUBIb	TTCCTTGTCCTGGATCTTCG*	

*

LuUBIa and LuUBI are published in (Huis et al. 2010).

Supplemental Table S3.2 Characteristics of the *Sorghum bicolor Pht1* gene family.

Gene name	JGI protein ID	Chromo-some	Strand	Length of genomic sequence (bp)	Length of genomic sequence without UTR (bp)	Length of cDNA sequence (bp)	Length of amino acids sequence (AA)	Number and length of introns (bp)	Trans-membran domains	Suppo rting ESTs	Best blast hit with *Phts* (% ID)	Pht1 signature GGDYPLSA TIxSE
SbPT1	5050188	1	+	1929	1569	1569	523	-	12	+	ORYsa;Pht1;2 (80)	GGDYPLSA TIMSE
SbPT2	5056998	6	-	1653	1566	1566	522	-	12	+	ORYsa;Pht1;4 (82)	GGDYPLSA TIMSE
SbPT3	5030542	1	+	1602	1602	1602	534	-	12	-	ORYsa;Pht1;8 (78)	GGDYPLSA TIMSE
SbPT4	5048813	1	-	1688	1569	1569	523	-	12	+	ORYsa;Pht1;3 (88)	GGDYPLSA TIMSE
SbPT5	5050189	1	-	1735	1608	1608	536	-	12	+	ORYsa;Pht1;12 (85)	GGDYPLSA TIMSE
SbPT6	5058886	7	-	2110	1626	1626	542	-	12	+	ZEAma;Pht1;3 (87)	GGDYPLSA TIMSE
SbPT7	5048812	1	+	2025	1626	1626	542	-	12	+	ZEAma;Pht1;1 (95)	GGDYPLSA TIMSE
SbPT8	5033142	2	-	1626	1626	1626	542	-	12	+	HORvu;Pht1;8 (78)	GGDYPLSA TIMSE
SbPT9	5041365	6	+	1533	1533	1284	416	1 (249)	12	+	ORYsa;Pht1 (70)	GGDYPLSA TIMSE
SbPT10	5041362	6	-	1638	1638	1512	504	1 (126)	12	+	TRImo;Pht1 (67)	GGDYPLSA TIMSE
SbPT11	5036358	3	-	1665	1665	1578	526	1 (87)	12	+	ZEAma;Pht1;6 (87)	GGDYPLSA TIMSE

Supplemental Table S3.3 Characteristics of the *Linum usitatissimum Pht1* gene family.

Gene name	Length of genomic sequence (bp)	Length of cDNA sequence (bp)	Length of amino acids sequence (AA)	Trans-membran domains	Best blast hit with *Phts* (% Identity)	Pht1 signature GGDYPLSA TIxSE
LuPT1	1870	1569	581	12	HORvu;Pht1;4 (76)	GGDYPLSA TIMSE
LuPT2	1786	1566	555	12	POPtr;Pht1;8 (74)	GGDYPLSA VIMSE
LuPT3	1802	1578	548	12	EUCca;Pht1;1 (77)	GGDYPLSA TIMSE

4 CARBON AND NITROGEN METABOLISM IN MYCORRHIZAL NETWORKS AND MYCOHETEROTROPHIC PLANTS OF TROPICAL FORESTS: A STABLE ISOTOPE ANALYSIS

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4.1 ABSTRACT

Most achlorophyllous mycoheterotrophic (MH) plants obtain carbon (C) from mycorrhizal networks and indirectly exploit nearby autotrophic plants. We compared overlooked tropical rainforest MH plants associating with arbuscular mycorrhizal fungi (AMF) to well-reported temperate MH plants associating with ectomycorrhizal basidiomycetes. We investigated ^{13}C and ^{15}N abundances of MH plants, green plants, and AMF spores in Caribbean rainforests. Whereas temperate MH plants and fungi have higher $\delta^{13}C$ than canopy trees, these organisms displayed similar $\delta^{13}C$ values in rainforests, suggesting differences in C exchanges. Although temperate green and MH plants differ in $\delta^{15}N$, they display similar ^{15}N abundances, and likely nitrogen (N) sources, in rainforests. Contrasting with the high N concentrations shared by temperate MH plants and their fungi, rainforest MH plants had lower N concentrations than AMF, suggesting differences in C/N of exchanged nutrients. We provide a framework for isotopic studies on AMF networks and suggest that MH plants in tropical and temperate regions evolved different physiologies to adapt in diverging environments.

4.2 INTRODUCTION

Mycorrhizal fungi symbiotically associate with plant roots and form networks between nearby plants through hyphal connections (Smith and Read 2008). Although there is increasing evidence for such links between plants of different species (Selosse et al. 2006), functional roles of mycorrhizal networks in plant physiology or ecosystem functioning remain debated. Whether carbon (C) transfer between plants occurs by way of mycorrhizal networks is particularly controversial (Bever et al. 2010, Courty et al. 2010). Nevertheless, myco-heterotrophic (MH) species are extreme and relevant models of plants that in most cases receive all their C through mycorrhizal networks (Leake 1994, Selosse and Roy 2009). These achlorophyllous plants live in the forest understory and exploit mycorrhizal fungi associated with surrounding autotrophic trees as a C and energy source (Taylor and Bruns 1997, Selosse et al. 2002, Bidartondo 2005, Roy et al. 2009) see (Martos et al. 2009, Ogura-Tsujita et al. 2009). These MH plants fully depend on the mycorrhizal network supported by autotrophic plants, and mycoheterotrophy opens a window to study of the properties of mycorrhizal networks. This work describes features of green plant-fungus-MH plant networks in overlooked examples from tropical ecosystems.

MH nutrition evolved many times independently in land plants, involving diverse plant and fungal taxa (Leake et al. 2004). In temperate ecosystems, MH plants mostly associate with basidiomycetes, as exemplified by orchids (Taylor et al. 2002) and Ericaceae (Bidartondo 2005). MH models from temperate regions were investigated using three major tools. First, molecular barcoding of mycorrhizal fungi demonstrated their association, often with high specificity, with fungi that are ectomycorrhizal on surrounding trees (Leake et al. 2004, Bidartondo 2005, Roy et al. 2009). Second, ^{14}C labeling of tree photosynthates demonstrated C transfer to MH plants (Björkman 1960, McKendrick et al. 2000). Third, stable isotope abundances were used as a major indicator to indirectly support the role of ectomycorrhizal fungi as a food source (Gebauer and Meyer 2003). Compared with their host trees, ectomycorrhizal fungi are enriched in ^{13}C and ^{15}N (Gebauer and Dietrich 1993, Hobbie et al. 2001, Mayor et al. 2009). In contrast, transfer from fungi to MH plants does not modify ^{13}C and ^{15}N abundances (or only slightly increases ^{15}N abundance; Trudell et al. 2003). In consequence, abundances of heavy isotopes are higher in MH plants than in cooccurring

green plants (Trudell et al. 2003). This pattern is also reported in some tropical forests where MH plants associate with ectomycorrhizal fungi (Roy et al. 2009). Isotopic abundances were also instrumental in the discovery that several green plants, phylogenetically close to MH species, obtain part of their C from ectomycorrhizal networks, since their ^{13}C and ^{15}N abundances are intermediate between autotrophic and MH plants (Gebauer and Meyer 2003, Julou et al. 2005, Tedersoo et al. 2006, Roy et al. 2009).

This nutrition is called partial mycoheterotrophy, a kind of mixotrophy that mixes photosynthetic and MH nutrition (for review, see Selosse and Roy 2009).

However, most MH species live in tropical forests devoid of ectomycorrhizal fungi (Leake 1994, Smith and Read 2008). In these forests, some MH orchids associate with saprotrophic basidiomycetes (e.g. Martos et al. 2009, Ogura-Tsujita et al. 2009), but most MH lineages connect to another kind of mycorrhizal network, formed by arbuscular mycorrhizal fungi (AMF; Imhof 2009). AMF are obligate biotrophic fungi that belong to Glomeromycota and evolved mycorrhizal symbioses independently from Basidiomycetes (Smith and Read 2008, Sanders and Croll 2010). Few AMF - associated MH plants (AMF-MH) have been investigated so far, and studies have focused on mycorrhizal morphology (Imhof 2009) and identification of AMF partners (Bidartondo et al. 2002, Franke et al. 2006, Merckx and Bidartondo 2008). Two features limit the use of stable isotopes in AMF-MH. First, AMF can associate with C_3, C_4, or CAM plants, and may thus mix C sources that differ in ^{13}C abundance (Allen and Allen 1990b), whereas ectomycorrhizal fungi only associate with C_3 plants. Second, AMF produce spores without conspicuous organs such as the fruitbodies of basidiomycetes, and extraradical mycelium and spores of AMF are not easy to sample for isotopic measurements. Allen and Allen (1990b) and Nakano et al. (Nakano et al. 1999) analyzed ^{13}C abundances in spores of AMF associated with herbs: The ^{13}C abundance followed that of their C_3 or C_4 hosts, with some depletion (22‰ to 24‰ in $\delta^{13}C$). Isotopic abundances were recently measured in two tropical rainforest AMF-MH, *Burmannia capitata* (Burmanniaceae) and *Voyria aphylla* (Gentianaceae): They showed higher ^{13}C abundance (+5.5‰ in $\delta^{13}C$), but similar ^{15}N abundance compared with understory green plants (Merckx et al. 2010). Enrichment in ^{13}C for AMF-MH is paradoxical, since depletion is expected in AMF (Allen and Allen 1990b, Nakano et al. 1999). However, isotopic abundances in AMF and canopy trees, likely the main C donors

to forest mycorrhizal networks (Högberg et al. 1999), have not been investigated up to now. Our aim was to provide isotopic data for several points along the continuum between tropical plant (canopy and understory), AMF fungus, and AMF-MH. A precise understanding of this continuum is required before predictions can be made on partially MH species associated with AMF.

We focused on four AMF-MH species at five sites of a Caribbean rainforest harboring C_3 trees (Rousteau 1996, Bonal et al. 2000; see Supplemental Table S4.1). They belong to two families that independently evolved MH nutrition (two species each of Gentianaceae and Burmanniaceae; see Supplemental Figure S4.1), allowing a phylogenetic replication of our results. We extracted AMF spores from the soil to measure their ^{13}C and ^{15}N abundances, and we verified that they included the species colonizing AMF-MH. We provide here, to our knowledge, the first report of ^{13}C and ^{15}N abundances along an AMF network *in situ*, including MH plants. We did not detect significant differences in isotopic abundances between canopy leaves, AMF mycorrhizal roots, AMF, and AMF-MH; however, due to specific photosynthetic conditions, green understory leaves are considerably depleted in ^{13}C. We propose a general framework for the interpretation of isotopic studies on AMF-MH, including predictions of isotopic abundances to be expected in partially MH species associated with AMF. Moreover, AMF-MH unexpectedly showed lower nitrogen (N) contents than MH plants associated with basidiomycetes, suggesting some physiological differences in N nutrition. Thus, we speculate that, beyond superficial similarities, tropical AMF-MH and - mostly temperate - MH taxa associated with basidiomycetes have different nutritional physiologies that evolved in adaption to their respective environments.

4.3 RESULTS AND DISCUSSION

^{13}C abundances differ in canopy versus understory green leaves

Since isotopic abundances significantly differed at the five study sites for each sample category (H = 15.706, degrees of freedom [df] = 4, P , 0.001 and H = 27.143, df = 4, P , 0.001 for $\delta^{13}C$ and $\delta^{15}N$, respectively; Kruskal-Wallis test, see Supplemental Table S4.1), data were analyzed separately (Figure 4.1, A–E). Values for $\delta^{13}C$ were variable for canopy leaves, due to interspecific differences (see Supplemental Table S4.1 for detailed sampling at Sofaïa 1), as expected in tropical rainforests (Bonal et al. 2000, Buchmann et al. 2004), and values for $\delta^{15}N$ were in the usual range for rainforests (e.g. Högberg and Alexander 1995). Leaves of understory plants were significantly depleted in ^{13}C compared with canopy leaves at all sites (on average by 5.3‰, but there was no significant difference in $\delta^{15}N$ (Figure 4.1, A–E). An approximately 5‰ difference in ^{13}C between canopy trees and understory plants is common in rainforests, as well as in any dense forest, due to several factors (for review, see Buchmann et al. 2004, Lüttge 2008). First, high light conditions enhance photosynthesis and drier conditions reduce stomatal conductance: These two factors limit equilibration between leaf intercellular and atmospheric $^{13}CO_2$ and $^{12}CO_2$ concentrations in canopy leaves, thereby reducing the isotopic discrimination. Second, the depletion in understory plants is enhanced by incorporation of $_{13}C$-depleted CO_2 originating from soil respiration. To summarize, measured isotopic abundances in autotrophic plant leaves indicated a canonical tropical rainforest pattern.

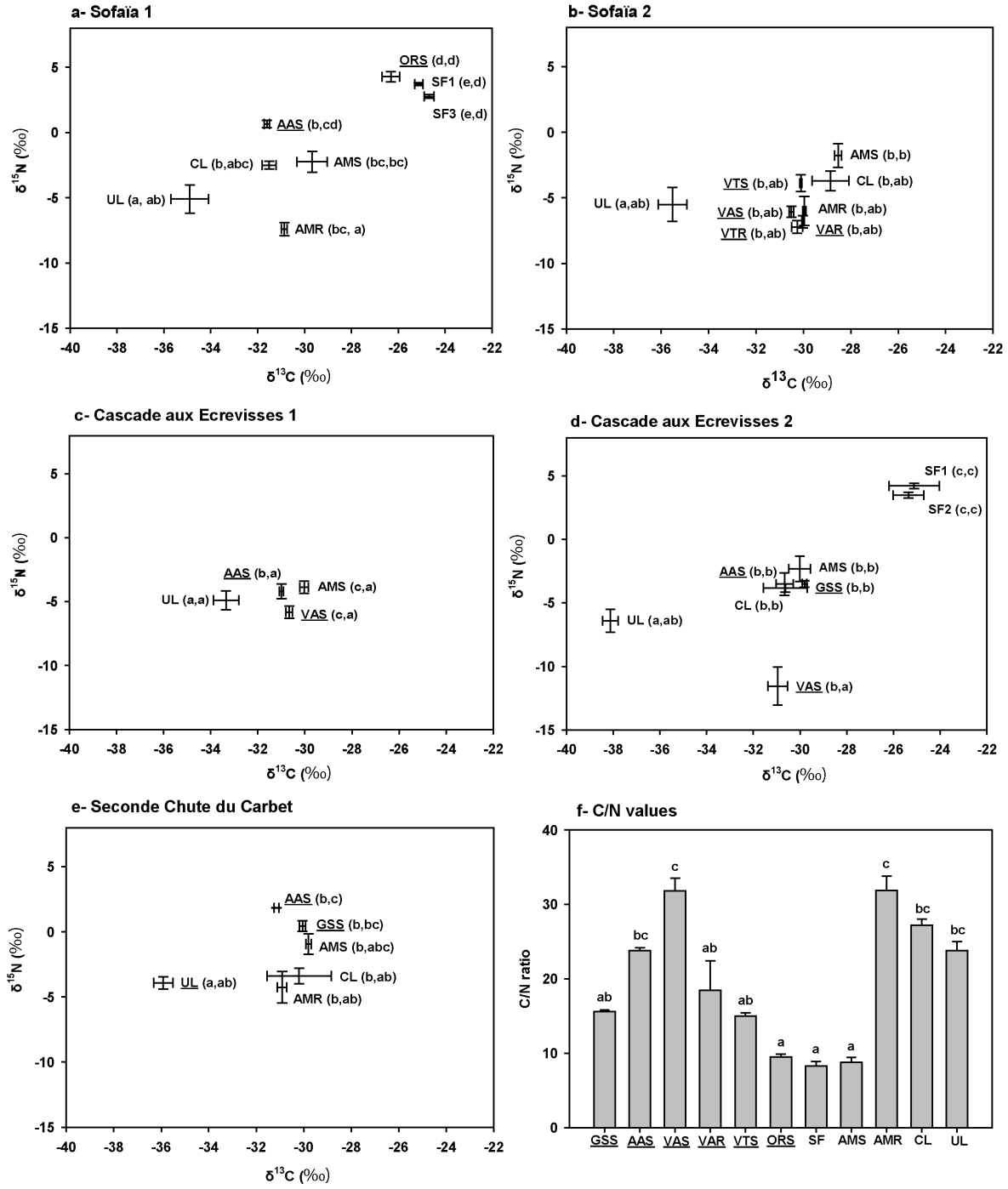

Figure 4.1. C versus N isotope abundance of plants and fungi at the five investigated sites (A–E) and N concentration (pooled for all sites; F), means ± se. Labels of AMF-MH organs are underlined. Abbreviations: AAS, shoots of the MH Burmanniaceae *A. aphylla*; AMR, fine roots of canopy trees mycorrhizal with AMF; AMS, spores of AMF; CL, green canopy leaves (see Supplemental Table S4.1 for species names); GSS, shoots of the MH Burmanniaceae *Gymnosiphon* sp.; SF, saprotrophic fungi (1: *Lycogalopsis solmsii*; 2: *Collybia* sp.; 3: *Marasmius* sp.); UL, green understory leaves (see Supplemental Table S4.1 for species names); VAR, roots of the MH Gentianaceae *V. aphylla*; VAS, shoots of *V. aphylla*; VTR, roots of the MH Gentianaceae *V. tenella*; VTS, shoots of *V. tenella*; ORS, shoots of the MH orchid *W. aphylla*. Different letters denote significant differences according to pairwise t tests (Bonferroni corrected, $P < 0.01$; in A–E, the first letter is for δ13C and the second one for δ15N).

AMF negligibly differ in isotopic abundances from canopy trees

AMF spores isolated from soil contained mixes of several species, as showed by barcoding on the internal transcribed spacer (ITS) of nuclear ribosomal DNA that provided several operational taxonomic units (OTUs; GenBank accession numbers HQ857159–91 and JF276256–74). Despite probable differences in species composition and proportions, independent replicates showed quite similar isotopic abundances at each site (differences between replicates, 0.5‰ in $\delta^{13}C$ and, 1.1‰ in $\delta^{15}N$, see Supplemental Table S4.1). Thus, we make the approximation, in this discussion, that AMF locally share a common isotopic abundance. AMF hyphae, which are the active transport pathways, may differ isotopically from AMF spores that are enriched in C reserves. However, in natural ecosystems, it is difficult to recover clean hyphae without cytoplasm loss by hyphal breakage. In addition, our results in simpler systems (sorghum [*Sorghum bicolor*] and flax [*Linum usitatissimum*] cultures in terragreen- sand mixture; F. Walder, unpublished data) have shown similar $\delta^{13}C$ values in AMF spores and hyphal networks. Thus, we assume here that isotopic abundances in spores are a good proxy for these in AMF mycelia.

Spores were higher in $\delta^{13}C$ than both understory and canopy leaves (Figure 4.1, A–E), but this difference was only significant for understory leaves. When they were available for analysis, mycorrhizal fine roots of canopy trees were also slightly, but non-significantly, more depleted in ^{13}C than AMF spores (by 0.96‰; Figure 4.1, A, B, and E). The observed ^{13}C enrichment in spores contrasts with the 2‰ to 4‰ depletion reported for spores of AMF colonizing temperate herbs, as compared with their host's roots (Nakano et al. 1999, on *Gigaspora margarita*) or roots and shoots (Allen and Allen 1990b, on mixed AMF); a similar approximately 2‰ depletion as compared to host was obtained in our sorghum (C_4) and flax (C_3) cultures mentioned above (F. Walder, unpublished data). A contribution of C_4 or CAM host plants could enrich AMF in ^{13}C (Nakano et al. 1999), but such plants were absent at our study sites. Since AMF displayed similar $\delta^{13}C$ differences with leaves and roots at all sites (Figure 4.1, A–E), this ^{13}C enrichment might be an idiosyncrasy of rainforest ecosystems. Indeed, as compared with previously studied temperate herbs, rainforest trees may differ by a higher flux of photosynthates to AMF (a higher flux would reduce the intensity of fractionation; Dawson et al. 2002); alternatively, tropical AMF may have lower levels of anaplerotic C

fixation: This pathway, which provides carboxylic acids to control cellular pH and/or assimilate N, often refixes respiratory CO_2 depleted in ^{13}C (Raven and Farquhar 1990).

Can a contribution of understory plants explain AMF $\delta^{13}C$? On the one hand, mixing canopy and depleted understory sources could lead to the observed $\delta^{13}C$ value in AMF if the latter receive compounds enriched in ^{13}C from all sources (see Supplemental Materials and Methods S4.1). Actually, carbohydrates transferred in plants from leaves to roots and likely to AMF are enriched in ^{13}C by approximately 3‰ in comparison with leaf bulk C (Gleixner et al. 1993). Detailed calculation (see Supplemental Materials and Methods S4.1) nevertheless shows that for enrichments up to 3‰, the major contribution (55%–100%) is of canopy origin. On the other hand, available data showing depletion in ^{13}C in AMF spores (Allen and Allen 1990b, Nakano et al. 1999; see above) is incompatible with this calculation, which assumes enrichment as compared with the plant source (see Supplemental Materials and Methods S4.1). If little or no depletion occurs, then the use of compounds from understory plants would reduce the $\delta^{13}C$ value in AMF as compared with canopy tree leaves and roots. Although the exact fractionation at the plant/AMF interface deserves further study, we thus conclude that, in all scenarios, canopy trees constitute the major C source for AMF. This is congruent with the higher photosynthetic activity of canopy leaves (Lüttge 2008), and with the situation reported in temperate regions, where forest ectomycorrhizal basidiomycetes receive 57% to 100% of their C from canopy trees (Högberg et al. 1999). Nevertheless, the observed isotopic pattern contrasts with ectomycorrhizal basidiomycetes from temperate forests, which are 1.2‰ to 2.9‰ enriched compared with canopy leaves (Högberg et al. 1999) and this suggests a different fractionation, or the transfer of isotopically distinct C compounds, during C exchange in AMF mycorrhizae and ectomycorrhizae.

AMF spores were significantly less enriched in ^{15}N than saprotrophic fungi (Figure 4.1, A and D). Even if considerable variation may occur depending on the N source (Gebauer and Taylor 1999), this contrasted with the ^{15}N enrichment usually reported in ectomycorrhizal fungi (Hobbie et al. 2001, Mayor et al. 2009). AMF were enriched in ^{15}N as compared with tree mycorrhizal roots (+3.01‰ on average) and all green leaves (+1.02‰), but often not significantly (Figure 4.1, A–E). This is less than the 0.5‰ difference in $\delta^{15}N$ between ectomycorrhizal fungi and host plant leaves in both temperate forests (Gebauer and Taylor 1999, Trudell et al. 2003, Tedersoo et al. 2006,

Selosse and Roy 2009) and tropical rainforests (Roy et al. 2009, Diedhiou et al. 2010). We think that these differences do not only result from tropical conditions, but mainly from physiological differences between AMF and ecto-mycorrhizal fungi. Although the pathways for N transfer to the plant are subject to debate (Chalot et al. 2006), they may not be fully identical in these two symbioses, leading to different fractionations. To summarize, to our knowledge, we provide the first measurements of $\delta^{13}C$ and $\delta^{15}N$ values for rainforest AMF in situ, and reveal negligible differences between AMF and canopy tree leaves. The reasons for these negligible differences deserve further physiological studies.

AMF-MH negligibly differ in isotopic abundances from AMF spores

ITS barcoding of mycorrhizal fungi revealed OTUs from a single *Glomus* group A clade in *Gymnosiphon* sp. at Seconde Chute Carbet (SCC) and *Apteria aphylla* at Cascade aux Ecrevisses 2 (CE2), as well as another clade in *V. aphylla* at Sofaïa 2 and CE2 (Figure 4.2; *Voyria tenella* was not investigated). At Sofaïa 2, *V. aphylla* displayed most of the OTUs from Glomus group A found at CE2 (11 out of 13), plus OTUs from four additional *Diversisporales* clades (Figure 4.2). Although barcoding on a single, highly polymorphic locus such as ITS has some limitations (Sanders and Croll 2010), our data are congruent with published evidence, based on ribosomal DNA coding sequences, that different AMF clades cooccur in *Voyria* species (Bidartondo et al. 2002, Merckx et al. 2010), and that several *Glomus* species occur in *Burmanniaceae* (Merckx and Bidartondo 2008, Merckx et al. 2010). Fungal specificity is often relaxed in tropical MH species (Martos et al. 2009, Roy et al. 2009), although the absence of fungal overlap between coexisting AMF-MH at CE2 is congruent with some partner preference. Beyond these corroborating results, AMF diversity in *V. aphylla* roots overlapped with that of AMF sporulating in soil at CE2 and Sofaïa 2: At least one OTU from each *V. aphylla* mycorrhizal clade was found, among other OTUs, in spores pooled from surrounding soil (Figure 4.2). Thus, ITS barcoding con- firms that AMF colonize our AMF-MH and that investigated soil spores partly originate from the mycorrhizal network linked to our AMF-MH. We did not further test whether the same OTUs occurred on the nearby tree roots, since it would be difficult to investigate the dense cluster of surrounding roots, but we consider that mycelia

sporulating or providing C to AMF-MH are necessarily connected with surrounding plants to support their C needs.

Shoots of cooccurring AMF-MH species did not significantly differ in $\delta^{13}C$, except at CE1, nor in $\delta^{15}N$, except at CE2 (Figure 4.1, A–E); reasons for these exceptions are unclear. Conversely, at Sofaïa 1, *A. aphylla* had sharply lower $\delta^{13}C$ and $\delta^{15}N$ values than the MH orchid *Wullschlaegelia aphylla*, which associates with saprotrophic basidiomycetes (Martos et al. 2009; Figure 4.1A). This supports the hypothesis of a similar nutrition for all AMF-MH, differing from the nutrition in other MH plants. AMF-MH shoots did not significantly differ from canopy leaves, AMF spores, and AMF roots for both $\delta^{13}C$ and $\delta^{15}N$ (with few exceptions, showing diverging trends; Figure 4.1, A–E). *A. aphylla, V. aphylla*, and *V. tenella* are on average somewhat depleted in ^{13}C compared to AMF spores, but this is not statistically significant. This situation resembles that for MH plants associated with basidiomycetes that share similar ^{13}C and ^{15}N abundances with their fungi (Gebauer and Meyer 2003, Trudell et al. 2003, Roy et al. 2009, Selosse and Roy 2009), as exemplified here by the MH *W. aphylla* and saprotrophic fungi.

At Sofaïa 2, the only site where enough material was recovered, AMF-MH roots did not differ isotopically from AMF-MH shoots and AMF spores (Figure 4.1B). As a result, AMF-MH shoots were significantly higher in $\delta^{13}C$ than understory leaves (on average by 4.8‰), but similar in $\delta^{15}N$ (or slightly higher at Sofaïa 1; Figure 4.1, A– E). Our finding corroborates data from a French Guiana rainforest, where shoots of the AMF-MH *Dictyostega orobanchoides* (Burmanniaceae) and *V. aphylla* were enriched in ^{13}C (by 5.5‰ and 5.4‰, respectively) but not in ^{15}N as compared with understory leaves (Merckx et al. 2010). However, having access to AMF spores, we demonstrate here that the difference in $\delta^{13}C$ is not driven by the AMF-MH metabolism or by $\delta^{13}C$ values in AMF (Figure 4.3), but that it likely arises from the photosynthetic conditions in canopy and understory trees, added to negligible changes in $\delta^{13}C$ along AMF networks. In this respect, AMF-MH plants contrast with MH plants associated with basidiomycetes, whose high $\delta^{13}C$ is driven by the ^{13}C enrichment of their mycorrhizal fungi.

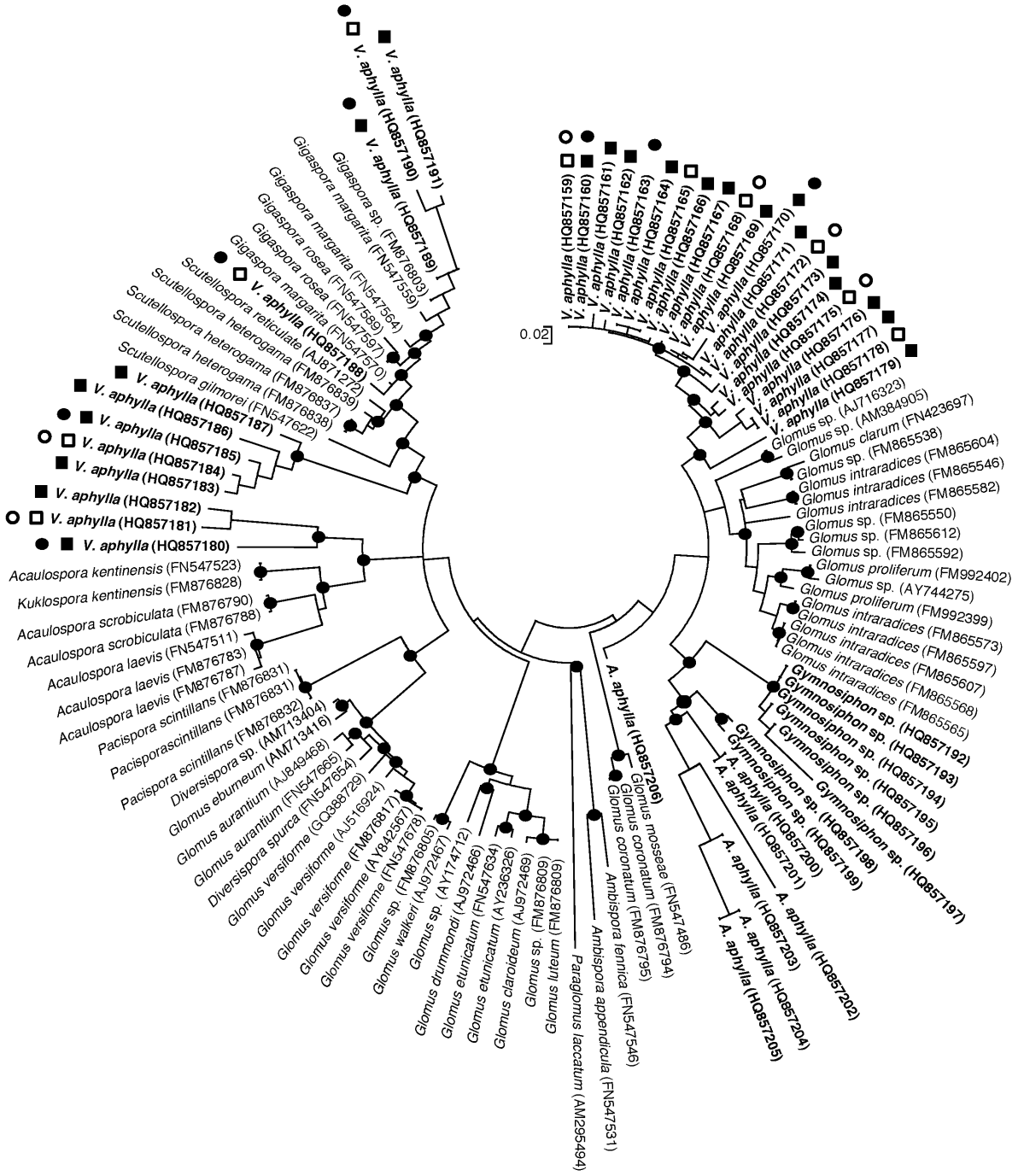

Figure 4.2. Neighbor-joining tree of AMF OTUs found in the roots of *A. aphylla* at CE2*, Gymnosiphon* sp. at SCC, and *V. aphylla* at Sofaïa 2 (squares) and CE2 (circles), based on an alignment of ITS sequences. For the later species, labels are white whenever the OTU was also found in spore pools from the soil surrounding *V. aphylla* and black otherwise. Nodes marked with a circle have bootstrap values >95% (from 5,000 replications); the bar represents 0.02 base substitutions per site.

Unexpectedly low N contents in AMF-MH

C and N concentrations did not differ among sites for each sample category (H = 2.157, df = 4, P = 0.707 for C and H = 2.327, df = 4, P = 0.676 for N; Kruskal-Wallis test, see Supplemental Table S4.1), and analyses at each site or on pooled data were congruent. C concentrations did not significantly differ, while N concentrations revealed some differences (Figure 4.1F): Together with saprotrophic fungi, AMF spores had significantly higher N concentrations than all other samples, except the MH orchid *W. aphylla* (see below). AMF-MH shoots showed nonsignificant differences in N concentrations between species (Figure 4.1F), which did not correlate with their systematic position, i.e. Gentianaceae versus Burmanniaceae. In *V. aphylla* and *V. tenella*, N concentrations were higher in roots than in shoots (Figure 4.1F), as expected since N-rich AMF colonize their roots.

N and C concentrations of AMF-MH were in the range typical for all green leaves (Figure 4.1F). Conversely, MH plants associated with basidiomycetes always have a higher N concentration than green plants (Gebauer and Meyer 2003, Julou et al. 2005, Roy et al. 2009) and tend to have reduced C concentrations (Stöckel et al. 2011), as exemplified here by the MH orchid *W. aphylla* (Martos et al. 2009; Figure 4.1F). The current interpretation for these MH plants is that they recover biomass from fungi with low C/N, and, by partly respiring it, further concentrate N (Abadie et al. 2006, Tedersoo et al. 2006, Stöckel et al. 2011). Moreover, high ^{15}N enrichment in these MH plants supports the hypothesis that they obtain fungal N through pathways differing from those in autotrophic plants (Gebauer and Meyer 2003, Selosse and Roy 2009). AMF-MH contrast with this scenario: First, to account for their moderate N enrichment after respiration, we have to suppose that they receive a fungal material with higher C/N than the average fungal biomass; second, as already stated by Merckx et al. (2010), similar ^{15}N enrichments in AMF-MH and green plants suggest that all tap isotopically similar N sources. To summarize, AMF-MH plants from two independent families, Gentianaceae and Burmanniaceae, differ from MH plants associated with basidiomycetes for mycorrhizal exchanges and N physiology, but are similar to green plants for N nutrition.

Beyond superficial similarities, such as reduced leaves or absence of stomata (Leake 1994), the different MH lineages evolved independently, each within different ecological contexts. It was suggested that MH plants associated with basidiomycetes, mostly from

temperate regions, might have arisen from a situation where C was simply hitchhiking with organic N and phosphorus (Selosse and Roy 2009). Mixotrophic plants phylogenetically related to MH support this hypothesis: these partially MH plants are considered to retain the ancestral situation predisposing to MH evolution. In temperate regions, some of them, such as the orchid *Limodorum abortivum* (Girlanda et al. 2006) or ericaceous relatives of *Pyrola* (Tedersoo et al. 2006, Zimmer et al. 2007), occur in environments where N is limiting, but not light. These plants may recover fungal compounds mainly as a source of mineral nutrients, including N (Selosse and Roy 2009). Even if C acquisition now plays the major role in MH plants associated with basidiomycetes, their high N content may have been inherited from this past functioning, persisting as an abuse of fungal N. Conversely, AMF-MH lineages probably arose in tropical forests (Merckx and Bidartondo 2008), where light is limiting, but not N (Hedin et al. 2009, Huston and Wolverton 2009). Thus, we speculate that tropical AMF-MH could have been selected directly for C acquisition, explaining why their N concentration and N nutrition are similar to those of surrounding plants. Thus, the convergence toward a similar MH nutrition may mask divergent histories and physiologies.

Implications for future research

We currently have only two isotopic studies on AMF-MH, all from South American rain forests (Merckx et al. 2010; this study); more broadly, research on tropical mycorrhizal symbioses should be encouraged (Alexander and Selosse 2009). More direct assessments of the fractionation at the interface between AMF and AMF-MH or autotrophic hosts are required, including analyses of transferred carbohydrates. Especially, the physiology of AMF-MH associations deserves the development of experimentally tractable AMF-MH models. Moreover, population-level, small-scale studies, using more marker loci than here, could rigorously demonstrate that AMF genets link AMF-MH to autotrophic hosts. In the future, investigations on the few available temperate AMF-MH (Bidartondo et al. 2002) could allow us to delineate which of the previous features are tropical, or strictly AMF dependent.

An exciting perspective is that some green species related to AMF-MH may be partially MH (Selosse and Roy 2009, Cameron and Bolin 2010, Merckx et al. 2010). Since AMF-MH are similar in ^{15}N and ^{13}C to their fungal sources, which are also similar to their own food source, isotopic enrichment is not a general fingerprint for MH nutrition. However, we predict that in dense forests at least, photosynthetic differences between canopy and understory plants (Figure 4.3) may result in ^{13}C differences between partially MH plants and surrounding understory plants. This may not apply to open forest or grassland ecosystems where all leaves receive similar light levels, entailing similar ^{13}C abundances, and where higher frequency of C_4 plants (Bonal et al. 2000, Lüttge 2008) complicates the use of ^{13}C abundances. Published investigations on candidate partially MH (=mixotrophic) plants associated with AMF showed that five green *Burmannia* species from French Guiana grassland (Merckx et al. 2010) and two Gentianaceae species from North American hardwood forest (Cameron and Bolin 2010) were not enriched in ^{13}C, or in ^{15}N (except for one Gentianaceae), as compared with surrounding ground plants. Given the higher light level in these ecosystems, this finding does not necessarily disprove partially MH nutrition in itself, and, indeed, Merckx et al. (2010) conclude compellingly that the ability of the *Burmannia* species to grow in pots, isolated from any mycelial network, demonstrates the possibility of full autotrophy. In any case, we recommend (i) joint analysis of isotopic abundances in AMF spores and leaves from understory and canopy (the most likely C providers), and (ii) the use, in dense C_3 forests only, of ^{13}C abundance for detecting partially MH plants.

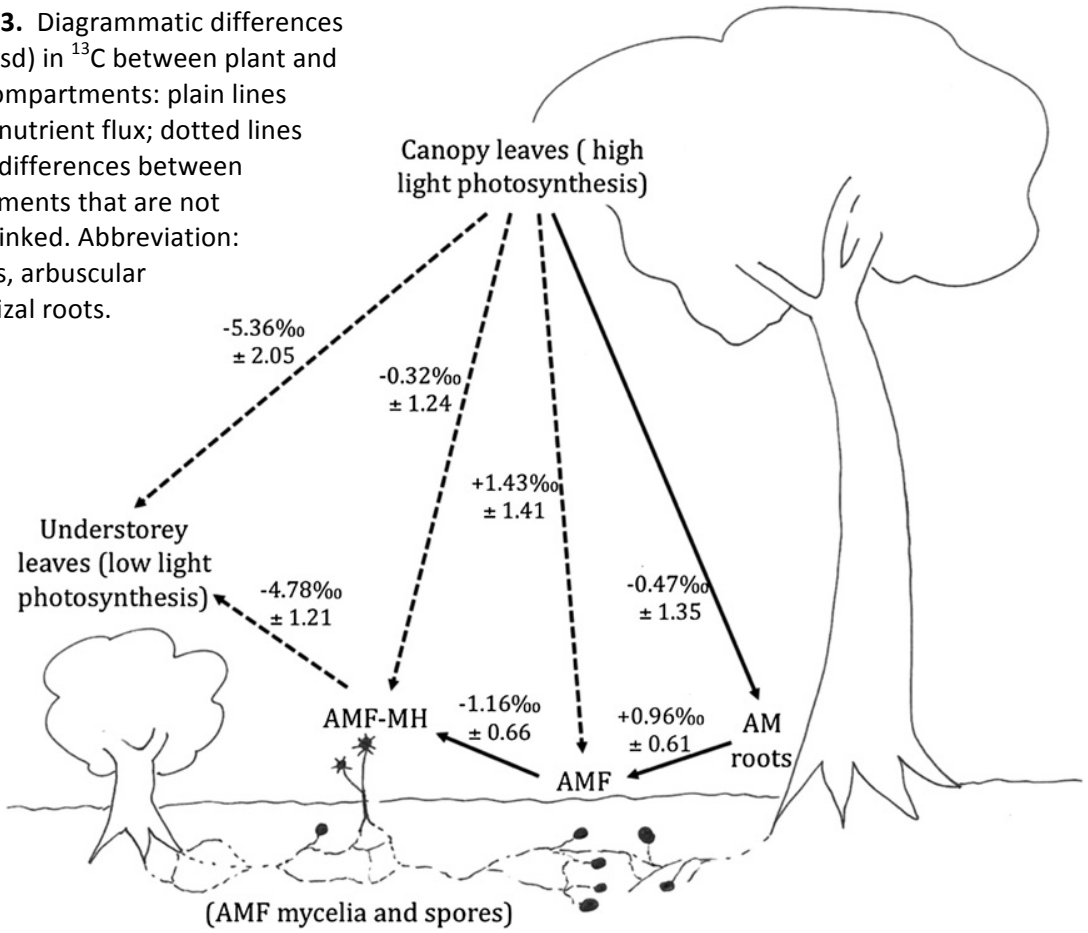

Figure 4.3. Diagrammatic differences (mean ± sd) in ^{13}C between plant and fungal compartments: plain lines indicate nutrient flux; dotted lines indicate differences between compartments that are not directly linked. Abbreviation: AM roots, arbuscular mycorrhizal roots.

4.4 CONCLUSION

This isotopic abundance analysis of AMF symbioses from rainforests offers a framework for understanding previous studies and planning future studies on AMF-MH and AMF networks. There is negligible isotopic change along the investigated points of the continuum between AMF (N providers to all partners), AMF-MH, and canopy trees (main C providers to all partners; Figure 4.3). Thus, all MH plants, associated with AMF or basidiomycetes, share similar ^{13}C and ^{15}N abundances with their mycorrhizal fungi, but high ^{13}C abundance as compared with green plants is not per se a general property of all MH plants. AMF networks differ from ectomycorrhizal ones by (i) a negligible difference in $\delta^{13}C$ between green plants (canopy trees) and fungi, (ii) the use of isotopically indistinct N sources by green and MH plants associated with AMF (Merckx et al. 2010), and (iii) a low N concentration in AMF-MH. The latter trend, found in two families that independently evolved AMF-MH species, suggests a different metabolism in AMF-MH as compared with MH plants associated with basidiomycetes.

4.5 MATERIALS AND METHODS

Study sites and sampling

Study sites are tropical rainforests along the wet eastern coast of the La Guadeloupe Caribbean Island. The forest harbors C_3 shrubs and canopy trees (Rousteau 1996, Sage 2001) and rare CAM epiphytes, likely not connected to AMF networks. Five sites (2 × 2 m; including three in the La Guadeloupe National Park) were sampled in the last week of December 2009 (late rainy season; see Supplemental Table S4.1 for samples and location). At these sites, the average total rainfall per year is > 2.33 m, and the average temperature is 24.7°C (ranging from 22.8°C–26.2°C in the coldest and hottest months). Near the Sofaïa spring (lowland tropical rainforest dominated by *Pouteria pallida, Guatteria caribaea*, and *Dacryodes excelsa*), we sampled two sites: Sofaïa 1, with the Burmanniaceae *Apteria aphylla* and the MH orchid *Wullschlaegelia aphylla* (associated with saprotrophic fungi; Martos et al. 2009), and Sofaïa 2 (200 m from Sofaïa 1), with the Gentianaceae *Voyria tenella* and *Voyria aphylla*. Near CE2 (tropical rainforest dominated by *Amanoa caribaea, D. excelsa*, and *Tapura antillana*), we sampled two sites: CE1, with *V. aphylla* and *A. aphylla*, and CE2 (150 m from CE1), with *V. aphylla, A. aphylla*, and the Burmanniaceae *Gymnosiphon* sp. (likely *Gymnosiphon sphaerocarpus*, endemic to the Caribbean islands)At SCC (transition between lower montane rainforest and montane thicket, dominated by *Amanoa caribaea, Richeria grandis,* and *T. antillana*), we sampled the same *Gymnosiphon* sp. and *A. aphylla* (see Supplemental Figure S4.1 for these AMF-MH). At all sites, we sampled soil, surrounding understory green plants (taking leaves at the height as from AMF-MH plants and avoiding young seedlings surviving on seed reserves). Whenever these were available, we collected fruitbodies of saprotrophic fungi and green canopy leaves mechanically torn off by heavy rains at the time of sampling (avoiding senescing leaves, and with a special sampling effort at Sofaïa 1, see Supplemental Table S4.1). Whenever roots of AMF-MH and/or fine roots of canopy trees mycorrhizal with AMF were found, they were carefully washed and kept separately. See Supplemental Table S4.1 for numbers of sampling repetitions. Samples were dried at 45°C for 48 h, except parts of AMF-MH roots that were kept at 280°C for molecular analyses.

AMF spore isolation

For each soil sample (see Supplemental Table S4.1), 30 g of dried soil was soaked and mixed for 30 min, and passed through 1,000-, 500-, 125-, and 32-mm sieves on a sieve shaker (AS 200 Digit; Retsch) with continuous shaking. The contents of the 125- and 32-mm sieves were resuspended in 20 mL of distilled water, and layered onto a LUDOX (HS-40 colloidal silica, 40 weight % suspen- sion in water, Sigma-Aldrich) solution gradient. After centrifugation (2,000 rpm for 2 min), spores were removed with a syringe, passed through a 32-mm sieve, and washed with tap water. Spores were then observed under a dissecting microscope, picked one by one, and transferred to a petri dish. A minimum of 200 spores were transferred into a zinc capsule and dried at 50°C for 24 h before isotopic analysis and a minimum of 100 spores were pooled for molecular analysis.

Molecular barcoding of AMF

We used the ITS of nuclear ribosomal DNA that is highly polymorphic, even within isolate (for review, see Sanders and Croll 2010): Although this may be a limitation for fungal identification, ITS is thus a sensitive marker for comparing AMF diversity in spores and AMF-MH roots. DNA was extracted from spore pools sampled under *V. aphylla* as well as roots of *V. aphylla, A. aphylla*, and *Gymnosiphon* sp. (n = 3–4 replicates per sample) using the NucleoSpin tissue KS kit (Macherey-Nagel) and amplified on a T3 thermocycler (Biometra). A first amplification was performed using SSUmAf and LSUmAr primer pairs as in (Krüger et al. 2009)on 1 mL of DNA extract in 12.5 mL final reaction volume. A nested PCR was then performed on 1 mL from the first amplicon, using the AMF-specific primers SSUmCf and LSUmBr as in (Krüger et al. 2009). For both PCR reactions, the FirePol DNA polymerase (Solis Biodyne; 0.5 units), 2 mM MgCl2, 0.5 mM each primer, and 0.25 mM each deoxynucleotide were used. Negative controls consisted of 1 mL of molecular grade water in all sets of PCR reaction to check for contamination and, in nested PCR, the second control consisted of a reamplified negative control from the first PCR round. Amplified fragments were subcloned into pCR2.1-TOPO and transformed into TOP10 Escherichia coli using the TOPO-TA cloning kit (Invitrogen). Individual colonies (24 per cloning) were screened for insert size by PCR amplification using M13 forward (5'-GTAAAACGACGGCCAGTG) and reverse (5'-GGAAACAGC-

TATGACCATG) primers. Amplified products were purified with ExoSAP treatment (USB) and DNA sequencing was performed on a 3500 genetic analyzer (Applied Biosystems), using the M13 forward and reverse primers, and ITS-3 (5'-GCATCGATGAAGAACGCAGC). Sequences were manually corrected using Sequencher 4.2 (Gene Codes). We applied a 3% divergence threshold to circumscribe OTUs, and consensus sequences were deposited in GenBank (accession nos. HQ857159–HQ857191 and JF276256–JF276274). To identify fungal species, BLASTN searches were carried out against the sequence databases at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Consensus sequences were aligned with additional GenBank sequences and a phylogenetic analysis was conducted under MEGA 4 (Tamura et al. 2007). A neighbor-joining tree was created for each genus from the alignment file using the Kimura 2-parameter method and bootstrapping of 5,000 replicates.

Isotopic analyses

All 184 investigated samples were ground in 1.5-mL Eppendorf tubes using 1.1-mm diameter tungsten carbide balls (Biospec Products, Inc.) in a Retsch MM301 vortexer (Retch GmbH and Co.). Total N and C concentrationsand abundances of 13C and 15N were measured using an online continuous flow CN analyzer (NA 1500; Carlo Erba) coupled with an isotope ratio mass spectrometer (Delta S; Finnigan). Isotope abundances are expressed in $\delta^{13}C$ and $\delta^{15}N$ values in parts per thousand relative to international standards V-PDB and atmospheric N_2: $\delta^{13}C$ or $\delta^{15}N$ = (Rsample/Rstandard 2 1) 3 1,000, where R is the isotope ratio, i.e. 13C/12C or 15N/14N. The SD of the replicated standard samples (n = 13 per each 100 samples) was 0.027‰ for ^{13}C and 0.245‰ for ^{15}N.

Statistics

N and C concentrations, as well as $\delta^{13}C$ and $\delta^{15}N$ values were tested for normality and homogeneity of variances using a nonparametric test (the Kruskal-Wallis test) as the distribution of replicates is not homogenous between sites. One-way ANOVAs were performed for each variable and each site, followed by a pairwise t test (Bonferroni corrected) to calculate pairwise comparisons between group levels at a = 0.01. All values were estimated by mean values followed by 95% confidence intervals. A general linear model procedure was used to calculate univariate ANOVA and to evaluate site effects on $\delta^{13}C$, $\delta^{15}N$, and C and N concentrations. Statistical analyses were computed using SPSS.Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers HQ857159 to HQ857191 and JF276256 to JF276274.

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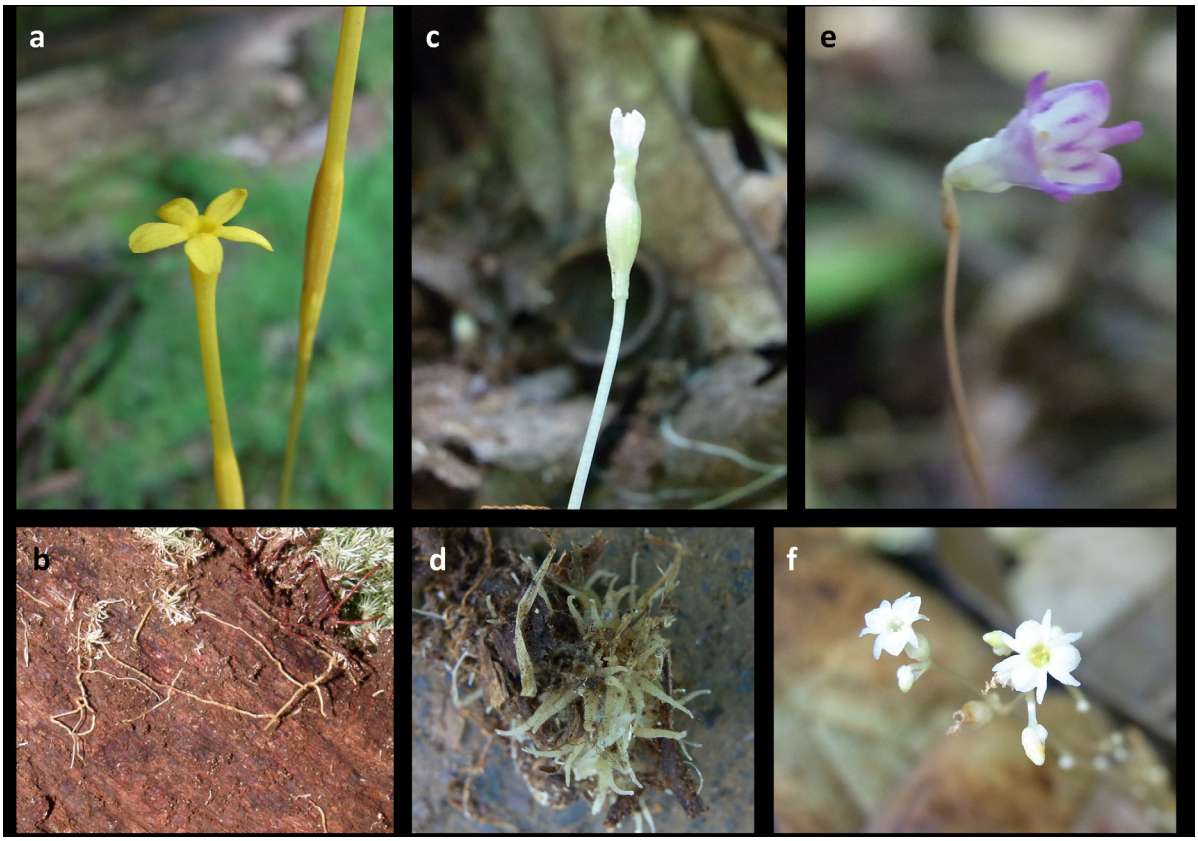
Table S4.1 The five study sites and samples investigated for isotopic abundances, as well as C and N concentrations. Abbreviations: AMF, arbuscular mycorrhizal fungi; AMF-MH, mycoheterotrophic plant associated with AMF.

Species and trophic status	n[a]	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	C/N	C%	N%
Sofaïa 1 (16°16'54.88"N; 61°42'58.68"O; elevation 279 m; rainfall: 2330 mm/yr)						
AMF-MH						
Apteria aphylla (Nutt.) Barnh. ex Small (shoot)	5	-31.60 ± 0.20	0.65 ± 0.48	22.28 ± 0.94	37.56 ± 0.12	1.45 ± 0.05
Mycoheterotrophic orchid						
Wullschlaegelia aphylla (Sw.) Rchb. f.	6	-26.31 ± 0.74	4.29 ± 0.77	9.50 ± 0.71	33.75 ± 1.82	4.79 ± 0.84
Understorey green leaves						
Scleria secans (L.) Urb.	1	-35.94	-2.58	35.56	31.22	0.75
Myrcia antillana McVaugh	1	-35.06	-4.92	23.80	31.31	1.13
Dacryodes excelsa Vahl	1	-32.56	-5.11	20.81	38.37	1.58
Asplundia rigida (Aubl.) Harl.	1	-36.02	-7.80	22.50	36.90	1.41
Canopy green leaves						
Pool of various sp. [b]	5	-29.77 ± 1.39	-3.70 ± 1.72	24.82 ± 5.02	36.11 ± 1.16	1.28 ± 0.10
Richeria grandis Vahl	4	-33.25 ± 0.09	-1.21 ± 2.73	24.56 ± 0.66	34.07 ± 0.47	1.19 ± 0.03
Dacryodes excelsa Vahl	5	-29.81 ± 0.50	-1.02 ± 2.00	33.55 ± 2.74	37.28 ± 0.25	0.96 ± 0.04
Rondeletia sp.	5	-29.71 ± 0.79	-1.23 ± 0.87	24.74 ± 3.82	37.34 ± 0.26	1.32 ± 0.12
Rudgea citrifolia (Sw.) K.	5	-34.94 ± 0.10	-1.84 ± 0.59	26.43 ± 1.91	32.71 ± 0.18	1.07 ± 0.04
Sapium caribaeum Urb.	5	-29.09 ± 0.25	-3.50 ± 0.32	17.96 ± 1.25	35.46 ± 0.07	1.70 ± 0.06
Endlicheria sericea Nees	4	-33.88 ± 0.05	-3.47 ± 0.59	22.97 ± 0.65	37.53 ± 0.13	1.40 ± 0.03
Ormosia sp.	5	-31.15 ± 0.08	-5.59 ± 0.31	31.90 ± 0.80	39.33 ± 0.15	1.06 ± 0.01
Licania ternatensis Hook. f. ex. Duss	4	-32.36 ± 0.47	-2.14 ± 0.97	33.72 ± 0.66	37.38 ± 0.30	0.95 ± 0.01
Pouteria pallida Gaertn. f. Baehni	4	-32.30 ± 0.98	-0.93 ± 3.00	38.19 ± 2.03	38.21 ± 0.72	0.86 ± 0.02
AMF						
Mycorrhizal tree roots [b]	5	-30.86 ± 0.21	-7.41 ± 0.85	30.92 ± 4.18	35.56 ± 1.72	1.01 ± 0.13
Spore pools	2	-29.67 ± 0.91	-2.25 ± 1.15	4.95 ± 0.65	32.64 ± 0.07	5.70 ± 0.73
Saprotrophic fungi						
Lycogalopsis solmsii E. Fisch.	4	-25.13 ± 0.34	3.71 ± 0.20	6.54 ± 2.20	33.75 ± 1.82	4.79 ± 0.84
Marasmiellus sp.	4	-24.68 ± 0.42	2.76 ± 0.28	7.99 ± 2.00	38.70 ± 2.24	4.34 ± 0.65

Sofaïa 2 (16°55'5.06"N; 61°42'58.80"O; elevation 284 m; rainfall: 2330 mm/yr)						
AMF-MH						
Voyria tenella Hook. (shoot)	4	-30.11 ± 0.09	-3.89 ± 1.30	14.99 ± 0.86	34.81 ± 0.19	1.99 ± 0.07
Voyria tenella Hook. (root)	2	-30.03 ± 0.05	-6.83 ± 0.65	14.51 ± 1.61	39.00 ± 0.42	1.28 ± 0.10
Voyria aphylla (Jacq.) Pers. (shoot)	4	-30.51 ± 0.19	-6.07 ± 0.84	32.88 ± 5.67	38.62 ± 0.37	1.03 ± 0.11
Voyria aphylla (Jacq.) Pers. (root) [c]	2	-30.71 ± 0.06	-8.05 ± 0.93	26.23 ± 2.41	39.88 ± 1.10	1.99 ± 0.41
Understorey green leaves						
Philodendron scandens C.Koch & H.Sello	1	-35.01	-5.16	23.44	33.94	1.24
Unknown sp. 1	1	-34.06	-4.51	22.54	37.91	1.44
Asplundia rigida (Aubl.) Harl.	1	-36.78	-9.14	28.51	33.95	1.24
Scleria secans (L.) Urb.	1	-36.23	-3.20	25.78	33.28	1.11
Canopy green leaves						
Pool of various sp. [b]	5	-28.86 ± 1.72	-3.71 ± 1.69	22.61 ± 6.25	34.04 ± 1.41	1.36 ± 0.14
AMF						
Mycorrhizal tree roots [b]	4	-29.96 ± 0.13	-6.00 ± 2.21	34.50 ± 12.57	38.99 ± 1.30	1.03 ± 0.11
Spore pools [c]	3	-28.54 ± 0.26	-1.78 ± 1.55	9.28 ± 1.50	43.80 ± 0.71	4.12 ± 0.46
Cascade aux Ecrevisses 1 (CE1; 16°10'42.06"N; 61°40'51.69"O; elevation 212 m; rainfall 3630 mm/yr)						
AMF-MH						
Voyria aphylla (Jacq.) Pers. (shoot)	3	-30.66 ± 0.24	-5.82 ± 0.85	33.38 ± 2,79	38.55 ± 0.41	1.03 ± 0.11
Apteria aphylla (Nutt.) Barnh. ex Small (shoot)	4	-31.00 ± 0.17	-4.19 ± 1.17	23.14 ± 0.86	37.81 ± 0.25	1.40 ± 0.03
Understorey green leaves						
Elaphoglossum sp.	2	-34.78 ± 0.59	-5.85 ± 2.50	24.96 ± 3.46	32.75 ± 1.57	1.13 ± 1.10
Dacryodes excelsa Vahl	2	-33.30 ± 1.59	-3.45 ± 1.35	24.99 ± 5.64	38.69 ± 0.82	1.37 ± 0.34
Ichnanthus pallens (Sw.) Munro	1	-33.70	-2.85	16.32	32.34	1.70
Unknown sp. 2	2	-31.78 ± 0.11	-6.35 ± 0.56	36.20 ± 2.16	40.82 ± 0.18	0.97 ± 0.05
AMF						
Spore pools	2	-30.04 ± 0.26	-3.87 ± 0.69	8.26 ± 4.30	39.25 ± 6.81	4.50 ± 1.63

Cascade aux Ecrevisses 2 (CE2; 16°10'41.68"N; 61°40'51.72"O; elevation 204 m; rainfall 3630 mm/yr)						
AMF-MH						
Apteria aphylla (Nutt.) Barnh. ex Small (shoot) [c]	5	-30.63 ± 0.63	-3.32 ± 1.60	24.83 ± 0.62	37.32 ± 0.17	1.29 ± 0.02
Gymnosiphon sp. (shoot)	2	-29.82 ± 0.15	-3.61 ± 0.36	15.02 ± 0.23	34.46 ± 0.21	1.92 ± 0.03
Voyria aphylla (Jacq.) Pers. (shoot) [c]	5	-31.02 ± 0.74	-10.40 ± 3.64	30.02 ± 7.95	37.71 ± 0.39	1.17 ± 0.22
Understorey green leaves						
Elaphoglossum sp.	1	-37.70	-8.89	45.26	33.64	0.64
Dacryodes excelsa Vahl	1	-39.06	-6.39	26.39	32.60	1.06
Asplundia rigida (Aubl.) Harl.	1	-37.66	-5.71	24.18	38.31	1.36
Scleria secans (L.) Urb.	1	-38.07	-4.64	25.77	33.47	1.11
Canopy green leaves						
Pool of various sp. [b]	5	-30.23 ± 1.86	-4.12 ± 0.88	26.21 ± 7.14	36.41 ± 1.12	1.25 ± 0.09
AMF						
AMF spore pools [c]	3	-30.01 ± 0.80	-2.33 ± 1.73	10.45 ± 2.51	39.60 ± 1.87	3.36 ± 0.52
Saprotrophic fungi						
Lycogalopsis solmsii E. Fisch.	4	-25.12 ± 0.42	4.21 ± 0.42	7.48 ± 2.31	38.70 ± 1.85	4.69 ± 0.62
Collybia sp.	4	-25.35 ± 1.32	3.48 ± 0.43	11.00 ± 1.68	40.41 ± 1.91	3.18 ± 0.15
Seconde Chute du Carbet (SCC; 16°02'37.72"N; 61°38'26.52"O; elevation 615 m; rainfall 5580 mm/yr)						
AMF-MH						
Apteria aphylla (Nutt.) Barnh. ex Small (shoot)	2	-31.15 ± 0.14	1.84 ± 0.02	25.52 ± 0.42	38.68 ± 2.64	1.30 ± 0.05
Gymnosiphon sp. (shoot) [c]	4	-30.05 ± 0.26	0.45 ± 0.84	15.58 ± 0.49	34.87 ± 0.30	1.92 ± 0.03
Understorey green leaves						
Ichnanthus pallens (Sw.) Munro	4	-36.59 ± 0.92	-2.03 ± 1.32	13.24 ± 0.83	34.35 ± 0.26	2.23 ± 0.08
Asplundia rigida (Aubl.) Harl.	5	-37.05 ± 0.50	-5.67 ± 0.46	23.21 ± 2.21	32.00 ± 0.72	1.19 ± 0.08
Melastomataceae sp.	3	-34.81 ± 0.74	-3.92 ± 2.32	19.64 ± 1.69	28.20 ± 0.68	1.24 ± 0.08
Dacryodes excelsa Vahl	3	-34.24 ± 2.21	-3.50 ± 0.76	22.35 ± 2.65	40.86 ± 2.71	1.59 ± 0.21
Erythrodes sp.	1	-38.21	-1.14	19.68	33.48	1.41
Canopy green leaves						
Pool of various sp. [b]	5	-30.20 ± 3.02	3.39 ± 1.32	27.91± 6.10	36.93 ± 0.65	1.18 ± 0.14
AMF						
Mycorrhizal tree roots [b]	2	-30.91 ± 0.29	-4.25 ± 1,70	31.69 ± 0.02	40.85 ± 0.59	1.10 ± 0.01
Spore pools	4	-29.79 ± 0.22	-0.94 ± 1.54	9.25 ± 1.07	43.76 ± 1.24	4.09 ± 0.26

[a] number of replicates; [b] each sample is a mix of 5 independent pieces, mixed in equal amounts; [c] samples used for AMF identification



Supplemental Figure S4.1 The four AMF-MH species under study. *Voyria aphylla* (a, flowers, and b, rhizomes) and *Voyria tenella* (c, flowers, and d, roots) are Gentianaceae. *Apteria aphylla* (e, flowers) and *Gymnosiphon* sp. (f, flowers) are Brumanniaceae.

Supplemental Method S4.1. Mixing model for $d^{13}C$ of AMF biomass, assuming a contribution of canopy and understory sources and an isotopic enrichment as compared to these plant sources.

Let us assume that the $\delta^{13}C$ value in AMF spores, S (in ‰), is the result of a mix of

- a proportion x from canopy source, whose $\delta^{13}C$ is C (in ‰),

- a proportion (1-x) from understory source, whose $\delta^{13}C$ is U (in ‰).

If we assume a positive enrichment (E, in ‰) when plants provide compounds to AMF, a linear mixing of the two sources can be written as:

$$S = x\,(C+e) + (1-x)\,(U+E) \quad \Rightarrow \quad x = (S-U-E)/(C-U)$$

Thus, using the values from Fig. 3, $x = (5.94-E)/5.36$

Note that to fit realistic situations, $0 \leq x \leq 1$; thus, using the values from Fig. 3:

- $X \leq 1$ entails that E should be above 0.59
- $X \geq 0$ entails that E should below 5.94

If E=0.59‰, then x = 1

If E=1‰, then: x = 0.92

If E=2‰, then x = 0.73

If E=3‰, then x = 0.55

Thus, canopy contribution is likely to be the major one, except if the enrichment E is extremely high.

5 TRACKING THE CARBON SOURCE OF ARBUSCULAR MYCORRHIZAL FUNGI COLONIZING C_3 AND C_4 PLANTS USING STABLE CARBON ISOTOPE RATIOS ($\delta^{13}C$)

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5.1 ABSTRACT

Arbuscular mycorrhizal fungi (AMF) may colonize several plant species simultaneously, thus receiving their carbon from different plants. In previous work, we have used microcosms with flax (a C_3 plant) and sorghum (a C_4 plant), connected to a common mycorrhizal network, in order to track the carbon source of AMF, making use of the distinct $^{13}C/^{12}C$ isotope compositions of C_3 and C_4 plants (Walder et al., 2012). Here we compare three methods for analysing the stable carbon isotope composition of AMF. Bulk carbon isotope analysis of washed extraradical mycelium is possible, but has the drawback of potential contamination from non-mycorrhizal sources. Bulk carbon isotope analysis of isolated AMF spores yields more reliable results but is rather tedious. We explain, in some detail, a more refined analysis based on the extraction of lipids from soil, followed by analysis of an AMF biomarker, the fatty acid C16:1ω5.

5.2 SHORT COMMUNICATION

A majority of plants on Earth live in a mutualistic symbiosis with arbuscular mycorrhizal fungi (AMF) of the phylum Glomeromycota (Smith and Read, 2008). AMF are obligate symbionts consuming up to 20% of their host plant's photosynthetically-fixed carbon (Bryla and Eissenstat, 2005); in return, they forage the soil with their extraradical mycelia (ERM) and deliver mineral nutrients to their host plants (Smith and Read, 2008). The ERM is a dense hyphal network, which acts as an extension of the plants' root systems and is often the largest contributor to the total microbial biomass in soils (Leake et al., 2004). Since AMF display little host specificity, the ERM of a single individual of AMF may form a common mycorrhizal network (CMN) with several different plant species (Bever et al., 2010).

In recent work, we examined the terms of trade in such a CMN in a model system consisting of two plant individuals growing side-by-side in compartmented microcosms (Walder et al., 2012). In these systems, we supplied ^{15}N and ^{33}P, in a compartment accessible only to the ERM, to trace the delivery of mineral nutrients to the plants. We used flax (*Linum usitatissimum*, a C_3-plant) and sorghum (*Sorghum bicolor*, a C4-plant), which display distinctly different ratios of $^{13}C/^{12}C$, to trace the delivery of carbon to the fungus. In our experimental setup, both plants had a similar capacity to fix carbon and to accumulate biomass when associated with a CMN in a mixed culture. However, we found a strong asymmetry in the terms of trade: flax gained up to 94% of the nitrogen and phosphorus provided by the CMN and grew much better than in the absence of a CMN, but invested little carbon. In contrast, the neighbouring sorghum received little nitrogen and phosphorus, but invested massive amounts of carbon into the CMN (Walder et al., 2012).

In the present article, we compare three different methods to assess the ^{13}C signature of carbon invested into the CMN, and we show that the analysis of the ^{13}C signature of the AMF biomarker fatty acid (FA) C16:1ω5 (Allen and Allen, 1990; Staddon et al., 1999; Olsson and Johnson, 2005), as shown in our previous work (Walder et al., 2012), is particularly suitable because it avoids much of the potential distortion of the ^{13}C signal by soil contaminants.

We performed three consecutive experiments in our microcosm system. The main results of two of them have been reported already (Walder et al., 2012). Briefly, microcosms were established with a 21 µm nylon mesh separating root compartments and a hyphal compartment, and filled with a substrate mixture consisting of TerraGreen, sand and Loess (5:4:1, w:w:w). The two host plants, a C3 plant (flax; *L. usitatissimum* L.) and a C4 plant (sorghum; *S. bicolor* (L.). Moench), were cultivated with and without mycorrhizal inoculation (about 100 spores of the AM fungus *Glomus intraradices*). Plants were grown as a pair of identical or as a pair of different plant species, in mono- or mixed cultures.

Table 5.1. δ ^{13}C values of AMF, assessed by different methods, and of flax and sorghum roots.

Object studied	Treatment	Experiment 1* ERM hyphae	Experiment 2 AMF spores	Experiment 3* Bulk soil, C16:1ω5
Flax (C_3)	NM	-32.48 ± 0.06$_a$	-32.58 ± 0.11$_a$	-33.41 ± 0.67$_a$
(bulk root	FF	-32.10 ± 0.12$_a$	-32.53 ± 0.17$_a$	-33.88 ± 0.68$_a$
analysis)	FS	-31.77 ± 0.07$_a$	-32.55 ± 0.33$_a$	-33.82 ± 0.71$_a$
Sorghum (C_4)	NM	-13.68 ± 0.10$_b$	-14.33 ± 0.10$_b$	-16.48 ± 0.10$_b$
(bulk root	SS	-13.73 ± 0.02$_b$	-14.54 ± 0.04$_b$	-16.41 ± 0.04$_b$
analysis)	FS	-13.67 ± 0.05$_b$	-14.28 ± 0.13$_b$	-16.95 ± 0.13$_b$
AMF	FF	-26.92 ± 0.80$_c$	-33.93 ± 0.25$_a$	-35.77 ± 0.07$_a$
(different	SS	-13.22 ± 0.37$_b$	-15.28 ± 0.37$_b$	-17.65 ± 0.13$_b$
methods)	FS	-15.29 ± 1.19$_b$	-19.85 ± 1.19$_c$	-23.23 ± 0.81$_c$

Table shows d13C values of plant roots of flax and sorghum grown without mycorrhizal symbiosis (NM) or in association with Glomus intraradices monoculture of flax (FF), sorghum (SS), and mixed culture (FS), and of AMF, analysed by different methods. All the AMF samples were harvested in a hyphal compartment excluding contamination by roots and spores originating from the inoculum. Experiment 1; AMF hyphae were extracted from the hyphal compartment by wet-sieving (Johansen et al., 1996). Experiment 2; AMF spores were extracted from the hyphal compartment by a density gradient (Courty et al., 2011). (C) Experiment 3: The AMF biomarker FA C16:1u5 was analysed after extracting lipids from bulk soil of the hyphal compartment (Niemann et al., 2005). Data represent mean ` SEM (A and B, N 1⁄4 4; C, N 1⁄4 6). Statistical analysis was performed for each experiment separately. Different lowercase letters indicate a significant difference between all values in each row (P 0.05) according to Tukey's test.

* Data of experiments 1 and 3 have been taken from a previous publication, which addressed the unequal terms of trade between plants and fungi connected by a CMN (Walder et al., 2012).

In the first experiment (Walder et al., 2012), microcosms were harvested after 3 months of cultivation, fungal hyphae in the hyphal compartment were isolated by wet sieving (Johansen et al., 1996), dried and subjected to bulk mass spectrometric analysis. In the second experiment, newly reported here, microcosms were harvested only after 4.5 months to provide sufficient AMF spore biomass. AMF spores contained in the hyphal compartment were isolated from the soil by a technique based on density gradient centrifugation (Courty et al., 2011). Bulk ^{13}C isotopic analysis of plant and fungal samples were performed by continuous-flow isotope ratio mass spectrometry (IRMS; ANCA, Europa Scientific Ltd., Crewe, UK). In the third experiment (Walder et al., 2012), microcosms were harvested after 3 months of cultivation, and the entire soil of the hyphal compartment was extracted in organic solvents of decreasing polarity to recover a total lipid extract (Elvert et al., 2003; Niemann et al., 2005). The total lipid extract was saponified with a methanolic KOH solution, and the resulting fatty acids were extracted and methylated, and then analysed by gas chromatographye IRMS (GC–IRMS). This allowed determination of the ^{13}C signature of individual FA, including the AMF biomarker FA C16:1ω5. All stable carbon isotope ratios are reported in the conventional δ-notation with respect to the V-PDB (Vienna Pee Dee Belemnite) standard.

In all three experiments, as a control and reference, roots were harvested and subjected to bulk analysis of the carbon isotopes (Table 5.1). Flax roots and sorghum roots always displayed the expected $\delta\ ^{13}C$ values of ca. -33‰ and ca. -15 ‰, respectively. While there was a slight variation of the $\delta\ ^{13}C$ values from experiment to experiment, particularly in sorghum, there were no significant differences in the $\delta\ ^{13}C$ values between non-mycorrhizal and mycorrhizal roots. In particular, the $\delta\ ^{13}C$ values of the roots of plants grown in conspecific or heterospecific pairs did not differ significantly, indicating that there was no significant carbon transfer between sorghum and flax through the CMN (Table 5.1). This stands in contrast to other studies indicating that, under specific conditions, a carbon transfer between C_4 and C_3 plants may occur in the field, potentially mediated by the CMN (Fitter et al., 1998; Carey et al., 2004).

In the first experiment, hyphae of the ERM in the hyphal com- partment were isolated, washed and subjected to bulk analysis of carbon isotopes. As previously reported (Walder et al., 2012), the $\delta\ ^{13}C$ value for AMF grown in association with flax was consistently about 5 ‰ less negative than that of the flax roots, indicating either carbon

isotope fractionation on the transfer from the root to the ERM or, more likely, a contamination of the isolated ERM by some extraneous material with a less negative δ ^{13}C value. This contamination was not apparent in the ERM associated with sorghum, because of the similar δ ^{13}C signatures of plant and substrate (-15.14 ‰ ±0.43 SEM, *n*=3); however, it was well apparent in the ERM associated with flax. Interestingly, the hyphae of the ERM isolated from a mixed culture of sorghum and flax displayed a δ ^{13}C value that was not significantly different from the sorghum monoculture but 15 ‰ less negative than that of flax, indicating that the AMF received almost all of its carbon from sorghum (Walder et al., 2012).

In the second experiment, which we have not reported before, we harvested AMF spores from the hyphal compartment and analysed their bulk δ ^{13}C value, thus focusing on a well-defined AMF structure and minimizing potential sources of contamination. In earlier work, it has been shown that the δ ^{13}C value of AMF spores reflected the carbon isotopic composition of their C3 or C4 host plants, indicating that the fungi did not take up any carbon from other sources (Nakano et al., 1999). As expected, spores from hyphal compartments associated only with one plant species had almost the same δ ^{13}C value as the corresponding plant roots. Spores isolated from hyphal compartments connected to both flax and sorghum had a δ ^{13}C value that was ca. 4.5 ‰ more negative than those from sorghum but 14 ‰ less negative than those from flax, indicating that the spores had obtained the bulk of their carbon (ca. 75%) from sorghum.

In a third experiment, we extracted lipids from the bulk soil from the hyphal compartment, saponified the lipid extracts to release the FAs from di-and triglycerides and analysed the resulting fatty acids as FA–methylesters by GC-IRMS. It has been shown that FA C16:1ω5 is specific for AMF (van Aarle and Olsson, 2003). We presented the results of this experiment with respect to the AMF-specific biomarker FA C16:1ω5 previously (Walder et al., 2012), but show here the pertinent parts of the original GC chromatograms to validate this type of analysis (Fig. 5.1).

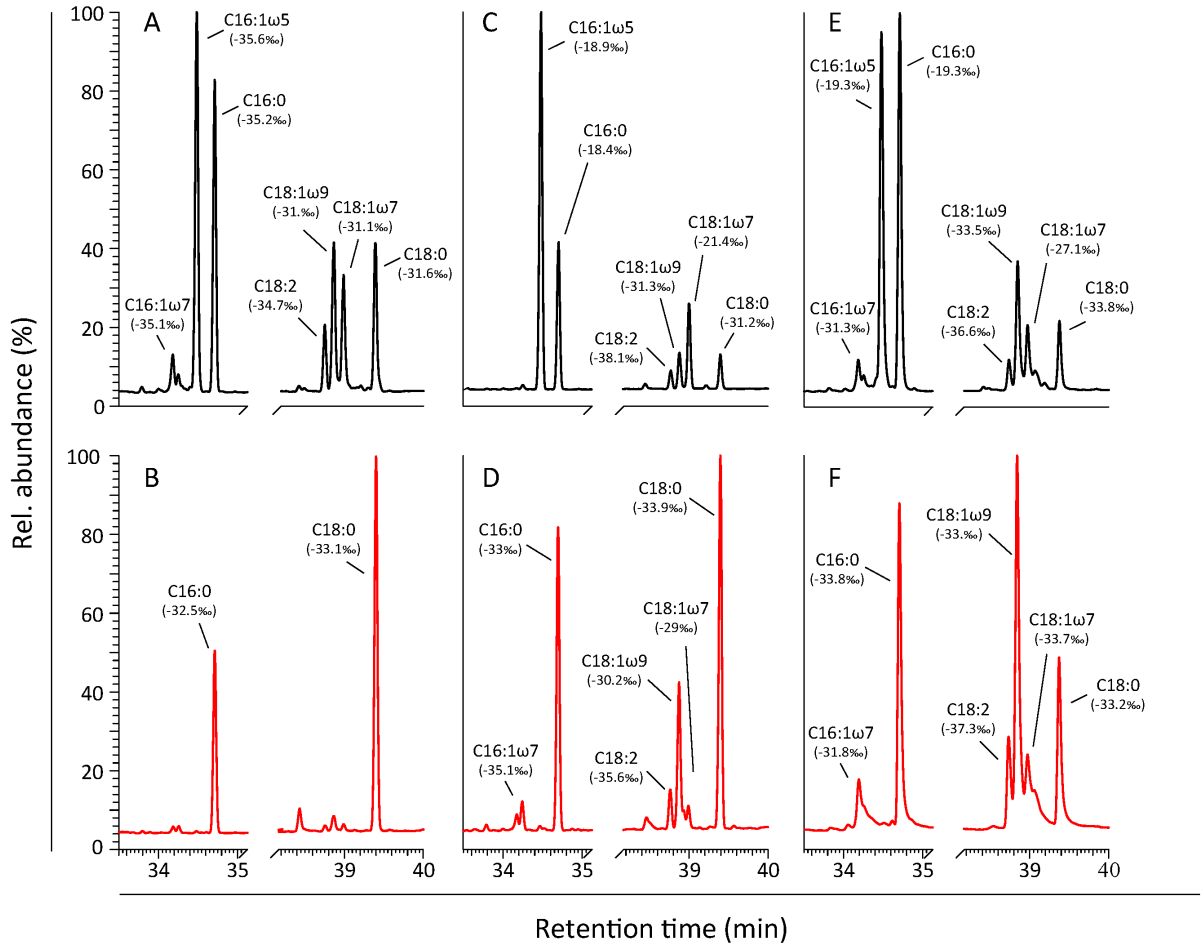

Figure 5.1 Partial gas chromatograms of fatty acids (analysed as fatty acid methyl esters e FAMES) extracted from hyphal compartments of microcosms populated with flax (*Linum usitatissimum*, a C_3 plant) and sorghum (*Sorghum bicolor*, a C_4 plant). Plants were grown in monoculture as pairs of identical plant species (Flax: A and B; sorghum: C and D) or in mixed cultures as pairs of different plant species (E and F), inoculated with Glomus intraradices (A, C and E), or with a non-mycorrhizal control (B, D and F). The identities of abundant peaks and their carbon isotopic signatures are indicated. The section of the chromatogram where C17-FAs elute is excluded from the graph because all samples/controls contained only minor amounts of these compounds. FA concentrations in the controls were about 30-fold lower compared to the inoculated systems.

Considering first the hyphal compartments of non-inoculated microcosms (Fig. 5.1B, D and F), the FA concentration was about thirty times lower than that in the ones of inoculated microcosms. The main FA were C16:0 and C18:0, two of the most common FAs in all organisms. Interestingly, these FA always had a $\delta^{13}C$ value of flax and sorghum (Table 5.1, Fig. 5.1E), the AMF biomarker FA C16:1ω5 had a $\delta^{13}C$ value that was ca. 4.5 ‰ more negative than that attached to sorghum but 14 ‰ less negative than that attached to flax, indicating, once more, that the hyphae had obtained the bulk of their carbon (ca. 75%) from sorghum.

In summary, $\delta^{13}C$ values of AMF material can be used to analyse the contribution of C3 and C4 plants to a common mycorrhizal fungal network. Analysis of the hyphae in the extraradical mycelium may be of interest because it is a relevant functional organ and has a high turnover rate, which guarantees for an immediate ^{13}C signal (Staddon et al., 2003). However, organic compounds adhering to the isolated hyphae, may be present as contaminants and distort the results (Table 5.1, Experiment 1). AMF spores are relatively easy to isolate and therefore pertinent to field studies as exemplified in an earlier study (Courty et al., 2011). However, a period of at least 3 months until sporulation is necessary to gain an adequate quantity of spores, which makes it less applicable for short-term experiments. Our best choice to track the carbon source of AMF is currently the $\delta^{13}C$ value of the AMF biomarker FA C16:1ω5. This FA may occasionally be found in bacteria (Joergensen and Wichern, 2008). However, at least in our model system it was exclusively present in microcosms with AMF, and its $\delta^{13}C$ value appears to reliably reflect the source of carbon, both in field and in experimental studies (Olsson et al., 1999).

Acknowledgements

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6 GENERAL DISCUSSION

6.1 DIVERSITY OF PLANT INTERACTIONS

The predominant plant ecosystems in temperate regions are forests and grasslands. Although they are completely different in their aboveground appearance, they have an invisible characteristic in common – both are dominated by mycorrhizal plants (Read 1991), which are interconnected by far-reaching mycorrhizal networks (Leake et al. 2004). In forests, trees are linked to ectomycorrhizal "wood-wide webs" while in grasslands networks are established by AMF - the so-called common mycorrhizal networks (CMNs). Neighboring plants do compete for light and resources, but also positive interactions such as facilitation play a key role in plant communities (Brooker et al. 2008). AMF have a great impact on plant coexistence (Hart et al. 2003). Indeed, mycorrhizal networks alter the competitive relation of neighboring plants by redistributing soil resources. In natural systems, plants are colonized simultaneously by several AMF species (Fitter 2005). A landmark study showed that this AMF diversity determines the diversity of a plant community (van der Heijden et al. 1998b). Remarkably, apart from diversity AMF also increase productivity of the plant community. The enhanced productivity indicates that there is not only a CMN-mediated shift in competitivity, but also that a CMN-mediated inter-plant facilitation takes place. Such positive interactions can occur when mycorrhizal networks are maintained by one plant while being beneficial to others.

In our model system, we were able to dissect the interaction of two plants (flax and sorghum) sharing an AMF and to describe the relationship between maintenance and beneficial service (Chapter 2). The growth of flax was facilitated by the adjacent sorghum by an uneven use of the CMN. Flax exploited the CMN for nutrients, but sorghum maintained the common symbiont by providing 70% of the invested carbon (C). Thus, the relation of nutrient return and carbon investment was very unfavorable for sorghum. From a plant-centric point of view, it is questionable how these unbeneficial terms of trade in the AM symbiosis could evolve in the AM symbiosis. However, it has to be considered that fungi developed survival strategies independently of those of the plants. Hence, the fungi may keep up the association with the plants even when these try to

chastise them under unfavorable conditions - especially in consideration of the obligate dependence on plants' C. However, sorghum exhibited no apparent negative effects when grown in the assumed to be disadvantageous association (Figure 2.3). This gives evidence that sorghum was not C limited and could cope with the additional C demand of the shared fungus. In agreement, a recent study showed that C is an excess, rather than a costly resource for plants associated to ectomycorrhizal fungi (Correa et al. 2012). Furthermore, sorghum is a C_4-plant equipped with efficient C allocation machinery and therefore probably produces a surplus of C compared to the adjacent C_3-plant flax. The exchange of such non-limited or luxury resources is thought to be the basis of trade in the AM symbiosis (Kiers and van der Heijden 2006).

Our experiment demonstrates that unequal terms of trade in the CMN increase besides the growth of the more benefited flax, also the overall productivity of the mixed culture system (Figure 2.2). Thus, our findings may also help to explain productivity and diversity promotion mediated by AMF found in plant communities (van der Heijden et al. 1998b, Wagg et al. 2011). Furthermore, traditional agricultural systems are usually composed of divers mixed cultures. Such biodiverse agro-ecosystems have been shown to be more efficient and often more productive than conventional monoculture systems (Perfecto and Vandermeer 2010, Hinsinger et al. 2011). The inter-plant facilitation found in these mixed cultural systems may also partially rely on the exchange of such luxury goods *via* the CMN.

6.2 TIT FOR TAT?

Mycorrhizal networks cannot be seen just as "social systems" distributing luxury resources between symbionts. Recent studies have shown that a plant individual simultaneously colonized by several AMF, rewards the more beneficial fungus by providing it with more C (Hammer et al. 2011, Kiers et al. 2011, Fellbaum et al. 2012). This is in accordance to a model describing an evolutionary stable strategy called "tit for tat", which was developed in the context of the Prisoners' Dilemma game (Axelrod and Hamilton 1981). In "tit for tat" the protagonists of an interaction cooperate on the first move and thereafter does whatever the opponent did on the previous move. This reciprocity in the interaction is thought to be generally valid for mutualistic associations with permanent contact. Nevertheless, such reciprocity of C investment and nutritional benefit has not been observed in our system comprised of two plants and one AMF (Figure 2.6). A recent study showed that two C_4 grasses have only a weak ability to reduce the C investment to non-beneficial fungus (Grman 2012). Thus, sorghum may not have the ability to sanction the non-beneficial association in our system. Sanctioning and rewarding of more or less beneficial AMF by the plant might be difficult anyway in case of the diffuse relations in CMNs; a fungus repressed by one plant may survive with resources from another plant individual (Kiers and Denison 2008). Anyhow, there is strong evidence for favored partnerships between plants and fungal symbionts in the AM symbiosis (Helgason et al. 2002), although the mechanisms regarding the maintenance of more beneficial partnerships remain obscure. Interestingly, the favored partnerships can even be extended to mycorrhizal networks interconnecting different plant groups (Montesinos-Navarro et al. 2012), which thereby form "guilds of mutual aid" (Figure 6.1; Perry et al. 1989). Such guilds may comprise plants of different functional groups, which profit from the mycorrhizal network by sharing complementary luxury resources such as nitrogen (N) fixed by legumes in symbiosis with rhizobia (Jalonen et al. 2009) or hydraulic-lifted water provided by deep-rooting plant species (Figure 6. 1; Egerton-Warburton et al. 2007).

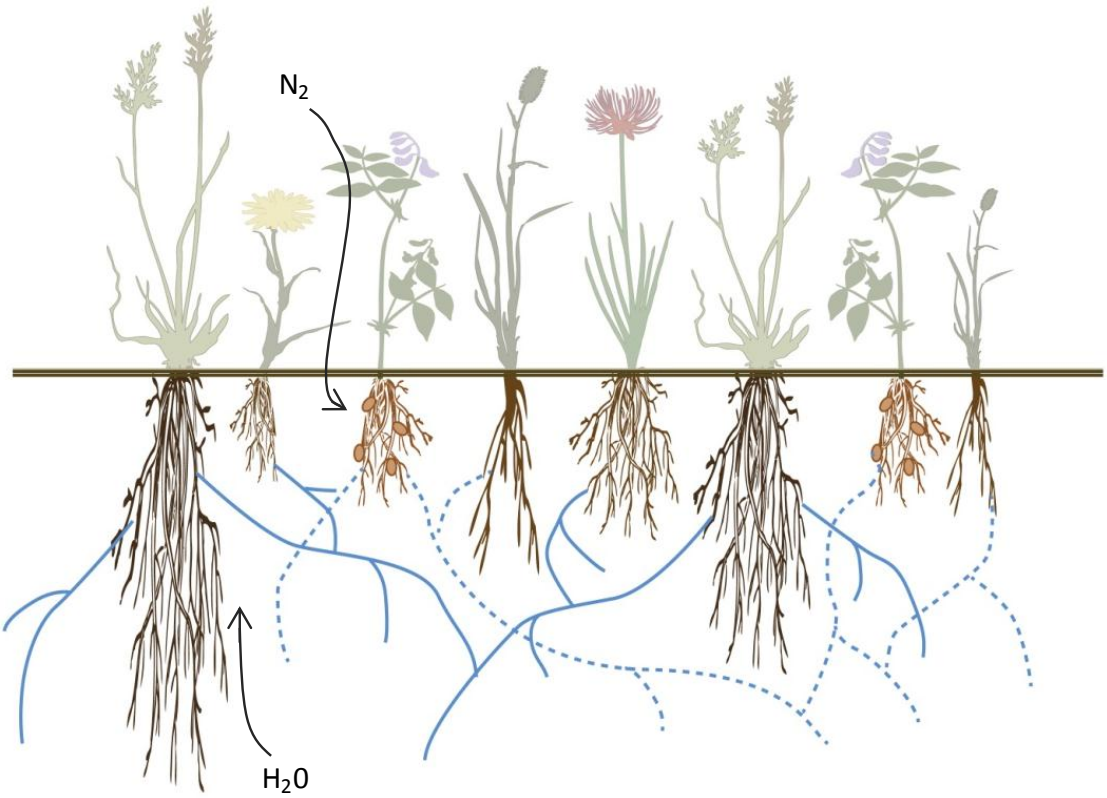


Figure 6.1 Different arbuscular mycorrhiza fungi (blue solid and dashed lines) interconnect plant groups *via* mycorrhiza networks and form thereby plant guilds of mutual aid. Such guilds may comprise plants of different functional groups, which profit from the mycorrhizal network by sharing complementary resources such as atmospheric nitrogen (N_2) fixed by legumes in symbiosis with rhizobia or hydraulic-lifted water (H_20) provided by deep-rooting plant species. The use of the resources offered in the mycorrhizal network may promote the growth of the whole plant guild.

Although, the relation of C investment and nutritional benefit appears to be not strictly reciprocal, the exchange of the plants C against mineral nutrients is an approved fact for autotrophic plants. Nevertheless, there also exists the extraordinary case in which the plant acquires both, nutrients and C *via* the AMF. In these associations the extent of the dependence of the plant on C supply from AMF ranges from additional C source besides photosynthesis in mixotrophic plants (Selosse and Roy 2009) to exclusive C source in mycoheterotrophic (MH) achlorophyllous plants (Chapter 4). In addition to the direction of C flux, the MH plants exhibit another exception in the AM symbiosis: MH plants show to a certain extent, specificity for AMF taxa (Figure 4.2). However, the role of the specificity in these partnerships is still undefined. Moreover, a recent study has shown

that the specificity may be weaker than assumed, because many MH plants are able to associate with a wide range of AMF (Merckx et al. 2012). Nevertheless, MH plants tend to be dependent on phylogenetically related AMF.

For MH plants, the terms of trade are obviously beneficial by acquiring beside nutrients also C *via* the CMN. Moreover, for the adjacent trees representing the primary source of C in the CMN the C loss is probably negligible (Figure 4.3). In contrast, for the AMF the association with mycoheterotrophic plants seems to have only negative effects. Interestingly, these are not antagonized although there is evidence that AMF are capable to penalize less beneficial plants. Studies conducted in root-organ cultures have shown that the C allocation of plants can trigger the release of phosphorus (P) into the mycorrhizal interface (Bücking and Shachar-Hill 2005, Hammer et al. 2011). When there is less C supply to the fungus, P is converted back to poly-P and the P concentration in the interface is reduced. Nevertheless, for unknown reasons AMF appear not to be able to inhibit the unfavorable associations with MH. A possible explanation could be the fact that MH actively digests their fungal partners (Imhof 1999).

6.3 RESOURCE EXCHANGE AT PLANT-FUNGAL INTERFACE

Both plants and AMF have the ability to reward and sanction their symbiotic partners, which should lead to certain reciprocity in the trade of resources in the AM symbiosis. Nevertheless, in our model system C investment and nutritional benefit were not related. Therefore, the exchange of resources might tend to be reciprocal in the AM symbiosis, but the outcome relies on different efficiencies between different plant-fungus combinations. Indeed, the ability of flax and sorghum to acquire nutrients available in the CMN was strongly dependent on the identity of the associated AMF (Figure 2.6).

The nutrient exchange in the AM symbiosis takes place at the plant-fungal interface comprised of specialized membranes of plant and fungus (Figure 1.2). Symbiosis specific ammonium and inorganic phosphate (P_i) transporters are transcriptionally induced and mediate the uptake of N and P across the periarbuscular membrane (Smith and Smith 2011). Thus, transcriptional regulation of such AM-inducible transporters may determine nutrient uptake *via* the mycorrhizal pathway. However, in our experiment the

expression of these AM-inducible P_i transporters of flax and sorghum were not related to the actual uptake of P_i available in the CMN (Chapter 3). This was most apparent in mixed culture with a CMN built by *G. intraradices*, where sorghum received virtually no P_i *via* the mycorrhizal pathway (Figure 2.5). Nonetheless, the expression of AM-inducible P_i transporters was not affected and still showed a highly induced expression level (Figure 3.4). This is surprising, as P_i uptake *via* the mycorrhizal pathway has been reported to be highly dependent on AM-inducible P_i transporters (Maeda et al. 2006, Javot et al. 2007a). Howsoever, the induction of these transporters appears to be rather dependent on the symbiotic association itself than on the symbiotic P_i uptake.

AM-inducible P_i transporters are crucial for the symbiotic P_i acquisition. But besides transcriptional regulation, the number of P_i transporters in the membrane is also determined by post-transcriptional mechanisms and protein turnover (Smith et al. 2003a). Furthermore, the extent of P_i transfer across the plasma membrane at the plant-fungal interface could also be dependent on other factors than the quantity of P_i transporters. For instance, the strength of a plant as a nutrient sink, in terms of plant size and nutrient concentration in plant tissue, has been considered to determine the ability of plants to exploit CMNs (Simard et al. 2002). However, sink strength can only partially be the reason, as we found strong differences for the same plant pair when it was connected by different fungal species (Figure 2.6). This is indicating that not only the sink strength of the plant, but also the identity of the AMF is affecting the outcome of nutrient acquisition in the CMN. In addition, the driving force for P_i transfer across the plasma membrane is the proton gradient generated by the H^+-ATPase (Karandashov and Bucher 2005). Therefore, the difference in P_i concentrations between the interconnected plant species plays only a subordinate role, at least on cellular level. Apart from the quantity of transporters present in the periarbuscular membrane, also qualitative characteristics of P_i transporters could influence the symbiotic P_i uptake. Different AM-inducible P_i transporters exhibit different functional features, such as different substrate affinities (Bucher 2007). Hence, the effectiveness of P_i transport by AM-inducible transporters might rely on such variable functional characteristics, which strongly affect the ability of plants to gain nutrients at the plant-fungal interface. Such differences between the AM-inducible transporters of flax and sorghum could be a further point, which helps to explain the observed differences in nutrient acquisition *via* the CMN.

Mycorrhizal P_i transfer across the periarbuscular membrane is surely dependent on P_i availability, hence the P_i concentration in the apoplastic space of the plant-fungal interface (Figure 1.2). Therefore, the release of P_i to the periarbuscular space by AMF partially defines the rate of P_i uptake by the plant. In order to reveal the role of fungal P_i transporter in the symbiotic P_i transfer, the expression of several P_i transporters genes of G*lomus intraradices* and *Glomus mosseae* were studied in the roots of sorghum and flax grown in mono- and mixed culture (Appendix A2, Figure A2.1). Remarkably, the expression of *GiPT4*, a homolog of Na^+/P_i symporter (PHO89) located in the plasma membrane of *Saccharomyces cerevisiae*, was substantially repressed in sorghum roots when sharing the fungal partner (*G. intraradices*) with flax. This is in accordance with the significant decrease in CMN-mediated P_i acquisition in this association (Figure 2.5). Unfortunately, the expression data of the homologous transporter in *G. mosseae* (GmPT4) is missing. An unrepressed expression of *GmPT4* in sorghum roots in mixed culture would be consistent with the ability to acquire nutrients from a CMN built by *G. mosseae*. This could partially confirm that PHO89 homologs play a crucial role in the regulation of fungal-plant P transfer. Nevertheless, our finding that *GiPT4* is repressed in *G. intraradices* associated to sorghum roots admits the hypothesis that the export of P_i to the periarbuscular space is decreased in sorghum roots. This is leading to the question why *G. intraradices* reduces the P_i transfer to the periarbuscular space. As sorghum was the main C provider for the AMF, it cannot be seen as a sort of punishment as it is described elsewhere for plants reducing C allocation to the fungus (Bücking and Shachar-Hill 2005). An explanation could be that the repression is triggered by the interconnected flax to enhance its own P_i supply. Even if this is rather speculative, it has been shown that interacting organisms can have a great impact on each others transcriptional phenotype (Whitham et al. 2003).

The fine-tuned exchange at plant-fungal interface has also been seen as basis of the reciprocal exchange in the AM symbiosis. Mainly the analysis of a mutant harboring the null allele of an AM-inducible P_i transporter in *Medicago truncatula* clearly demonstrated that P_i import can be crucial for the development of the AM symbiosis (Javot et al. 2007a). Thus, the transfer of P_i across the periarbuscular membrane might be a mechanism to distinguish beneficial from less beneficial AMF. In this spirit, P_i would act as a signal necessary for the maintenance of the symbiosis (Yang and Paszkowski 2011).

However, considering P_i as the exclusive signal determining the outcome of the interaction is too simple. The mycorrhizal P_i uptake was diminished in sorghum sharing the CMN with flax, but the symbiosis persisted, even though with reduced numbers of arbuscules in sorghum roots (Table S2.2). Furthermore, a recent study showed that the development of the symbiosis is not only P but also N triggered (Javot et al. 2011). Thus, the null allele mutant of the AM-inducible P_i transporter in *M. truncatula* exhibited a normal development of AM symbiosis under N limited conditions.

6.4 REPRODUCIBILITY OF THE FUNCTIONAL EXPERIMENT

In the process of developing appropriate methods to measure C investments to CMN, the same experimental set-up was repeated four times. In here the different experiments are referred to as experiment 07, 08, 09 and 10 according to the year of performance. The experiments 07, 08 and 10 were conducted following exactly the same set-up (for details see Chapter 2). The experiment 09 was conducted in the greenhouse with a slightly different compartment system that had no hyphal-label compartment (Figure 2.1). Additionally, the growth period was extended to 4.5 month to await sufficient spore production. In the experiments 07, 09 and 10, sorghum provided between 70% and 85% of total C invested into the CMN (Figure 2.3 and S2.2; Appendix A5, Figure A5.4). As an exception in the experiment 08, the C investment of flax and sorghum was similar (Appendix A4, Figure A4.4). The imbalance in nutrient uptake *via* the CMN was apparent in the experiments 07, 08 and 10, where flax received over 90 % and over 80 % of CMN-mediated P and N, respectively (Figure 2.5 and S2.2, Appendix A4, Figure A4.2 and A4.3). The exception was experiment 09, where sorghum acquired more CMN-mediated nutrients than flax (Appendix A5, Figure A5.2 and A5.3). In experiment 09, sorghum plants were more than three times bigger than flax plants (Appendix A5, Figure A5.1) while in the other experiments the difference in biomass was around the factor 1.5 (Figure 2.3 and Table S2.1; Appendix A4, Figure A4.1). Therefore, sorghum probably formed much a greater sink in terms of plant size, which may be resulting in a higher competitivity for CMN-mediated nutrients compared to the more size balanced interactions in the other experiments. If experiment 09 is not taken into account by

reason of the exceptional characteristics, the relative C investment appears to be more variable than the relative nutrient uptake. Hence, the C investment appears to be dependent on the surplus of C for the plants, while nutrient acquisition appears to be more dependent on the functional compatibility of the present plant-fungal interaction.

A comparison of the hyphal length densities in different experiments elucidates an interesting fact. In the mixed cultures of experiment 10, the density of the hyphal network was similar to the one in sorghum monoculture (Figure S2.3). This observation was even more pronounced in earlier experiments, where the densities of the hyphal network in mixed culture and monoculture of sorghum even showed no significant difference (Appendix A3 and A4, Figure A3.1 and A4.5). However, under the assumption that the extent of C investment of sorghum and flax are similar in mono- and mixed culture, the hyphal length density in mixed culture should be in between the densities of sorghum and flax monocultures. Hence, one of the two associated plants increased the C investment to the fungus, which according to the C signature of the mycorrhizal network has to be sorghum. Thus, flax seems to push sorghum to invest more C into the CMN.

6.5 PERSPECTIVES

In our model system, we were able to describe the exchange of resources between two plants and a shared CMN. Resource exchange in the compartment system can also be investigated in a higher resolution due to the high sensitivity of the analysis of AM-specific fatty acid (Chapter 5). More precisely, higher resolution can be achieved by dividing the hyphal compartments in several sectors, in which P_i uptake and C investment can be related. Such “high-resolution” measurements could reveal the role of the proximity of different plants in the terms of trade in CMNs. Furthermore, the model system can be extended by changing many parameters, such as size dimensions of the compartments, developmental stages of the plants (flax was flowering whereas sorghum was in a vegetative stage) and nutrient availability in the substrate (more controlled as with TerraGreen), which all could greatly affect the terms of trade in the CMN.

The same set-up can also be used to investigate other interactions. Besides C, N is a major component of plant and fungal tissues. Therefore, legumes fixing N in symbiosis

with rhizobia could provide N to the system, as indicated in earlier studies (Martensson et al. 1998, Jalonen et al. 2009). The luxury good N provided by the legume could facilitate the growth of the whole culture system similar to the C of sorghum in our experiments. This effect can be investigated in our model system by including a legume as one of the model plants. Moreover, in mixed culture systems including legumes and C_4-plants, the complementary exchange of surplus resources *via* CMNs can be expanded to investigations in field by measuring the C and N isotopic signature of AMF spores (Chapter 5). Such investigations can be done for example in mixed culture systems using the complementarity of legumes and C_4 grasses, as for example the famous system called "three sisters" comprised of squash, maize and beans (Postma and Lynch 2012), or in the mixed culture systems including faba bean and maize described in Li et al. (2007).

Besides the exchange of luxury goods, CMNs could mediate further inter-plant facilitative effects in mixed culture systems. For instance, different plant species grown together in mixed culture systems are often harvested sequently. Therefore, the roots of the first harvested plants represent resource rich pools in the soil, which can be used by the co-cultured plant. CMNs may enhance the access of the co-cultured plant to these resources by providing an inter-plant "short cut" *via* the already existing hyphal connections (Jakobsen 2004). Moreover, such hyphal inter-plant connections could also provide in the same way competitive advantages to plant guilds interconnected by CMNs (Figure 6.1). The loss of a plant individual, for example by grazing, would thereby generate a resource gain for the interconnected plant community. The resources of the aboveground grazed plant could be kept in the plant guild by avoiding competition for the resources with other organisms of the rhizosphere.

The use of the model system provides to a certain extent unnatural results as direct plant interactions including root competition were inhibited by the compartmented design of the microcosm. Still, such compartmented systems allow investigations on complex interactions of plant and AMF by separating them physically and functionally. Moreover, hyphal compartments only accessible for AMF represent the additional volume hyphae access in soil and therefore provide sort of natural conditions in terms of soil exploration in artificial systems. Nevertheless, follow-up studies with more nature-orientated designs could be of interest. For instance, sorghum and flax can be grown in a non-compartmented microcosm competing for nutrients by roots and AMF. By use of AM-

specific fatty acids, the C allocation to the CMN could still be tracked also without physical separation (Chapter 5). Possibly, sorghum will gain on competitive ability for nutrients due to enhanced direct root access, because it is equipped with a large and fibrous root system indicating independence of the association with AMF (Smith and Read 2008). However, the down-regulation of P_i transporters involved in the direct pathway (Chapter 3) could negatively affect the ability of sorghum to acquire P_i directly. Furthermore, the expression level of AM-inducible P_i transporters was only marginally connected to the functionality of the mycorrhizal P_i uptake. For a better understanding of the functionality of these transporters, investigations at the protein level would be necessary and could also shed light on the fine-tuning of the mycorrhizal pathway in relation to P_i availability in CMN.

In the end, C contribution to the CMN of different plants can be investigated in the field by looking at AMF spores (Chapter 5). For instance, this could help to reveal the C source of plants, which are thought to be mixotrophic (Selosse and Roy 2009).

6.6 CONCLUDING REMARKS

In this thesis we were able to show that C investments and nutritional benefits of different plants can be dramatically asymmetric in mycorrhizal networks. In the pairing of the two autotrophic plants, flax invested only little C but gained almost all nutrients provided, while sorghum invested massive amounts of C without receiving a corresponding benefit. An even absolutely asymmetric resource exchange displays the MH interaction, where achlorophyllous plants acquire not only nutrients but also their entire C *via* CMNs. Flax and heterotrophic plants appear to use the CMN without corresponding C contributions – hence they are cheating the associated partners. However, where cheating occurs, the victim should be found close-by. In the systems studied in this thesis no victims in the sense of apparent negative effects could be found. The main C provider in both associations was able to cope with the additional C drain caused by the cheaters. Without victims, no cheaters: the asymmetric exchange of resources is therefore probably not forced by the recipient organisms, but rather by the supply of surplus resources. Thus, cheaters of CMNs are rather opportunists effectively using the surplus resources of interconnected plants.

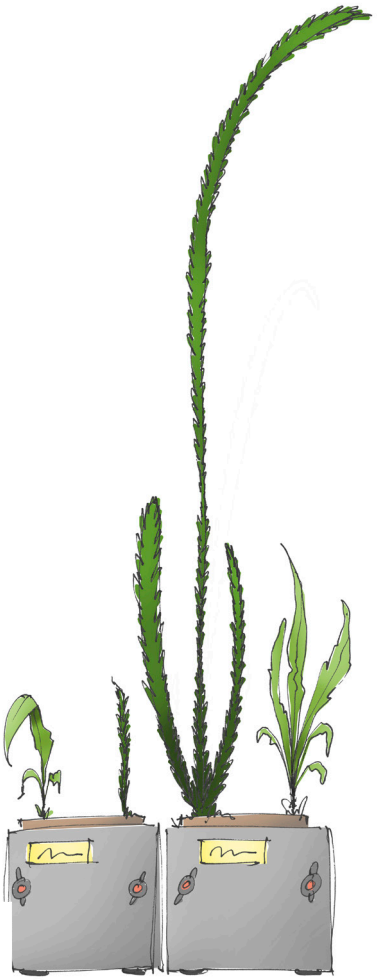


Illustration by Nils Fisch (TagesWoche)

7 APPENDIX

A1. Phosphate transporters

Table A1.1 Expression pattern and affinities of some fungal and plant phosphate (P_i) transporters

Organism	Official nomenclature[a]	Short name[b]	Expression pattern[c]	Affinity (Km)[d]	References[e]
Saccharomyces cerevisiae		PHO84		8 µM	1
Glomus versiforme		GvPT		18 µM	2
Glomus intraradices		GiPT			3
Glomus mosseae		GmosPT			4
Lycopersicon esculentum	LYCes;Pht1;1	LePT1		31	5, 6
L. esculentum	LYCes;Pht1;2	LePT2			6
L. esculentum	LYCes;Pht1;3	LePT3	AM +		6
L. esculentum	LYCes;Pht1;4	LePT4	AM S		6
L. esculentum	LYCes;Pht1;5	LePT5	AM +		6
Solanum tuberosum	SOLtu;Pht1;1	StPT1	AM -	280	6, 7, 8
S tuberosum	SOLtu;Pht1;2	StPT2	AM -	130	6, 8
S. tuberosum	SOLtu;Pht1;3	StPT3	AM +	64	6, 8, 9
S. tuberosum	SOLtu;Pht1;4	StPT4	AM S		6
S. tuberosum	SOLtu;Pht1;5	StPT5	AM S		6
Medicago truncatula	MEDtr;Pht1;1	MtPT1	AM -	192	10, 11
M. truncatula	MEDtr;Pht1;2	MtPT2	AM -		10, 11
M. truncatula	MEDtr;Pht1;4	MtPT4	AM S	668	9, 11
Lotus japonicus	LOTja;Pht1;1	LjPT1	AM -		12
L. japonicus	LOTja;Pht1;2	LjPT2	AM-		12
L. japonicus	LOTja;Pht1;	LjPT3	AM +		12
Glycine max	GLYma;Pht1;2	GmPT2		7	13, 24
G. max	GLYma;Pht1;3	GmPT3	AM -		13
G. max	GLYma;Pht1;4	GmPT4	AM -		13
G. max	GLYma;Pht1;5	GmPT5			13
G. max	GLYma;Pht1;6	GmPT6	AM -	7	13, 14
G. max	GLYma;Pht1;7	GmPT7	AM S		13
G. max	GLYma;Pht1;8	GmPT8			13
G. max	GLYma;Pht1;9	GmPT9	AM -		13
G. max	GLYma;Pht1;10	GmPT10	AM S		13
G. max	GLYma;Pht1;11	GmPT11	AM S		13
G. max	GLYma;Pht1;12	GmPT12	AM -		13
G. max	GLYma;Pht1;13	GmPT13	AM -		13
Populus trichocarpa	POPtr;Pht1;1	PtPT1	AM -		15
P.trichocarpa	POPtr;Pht1;2	PtPT2			15
P.trichocarpa	POPtr;Pht1;3	PtPT3			15
P.trichocarpa	POPtr;Pht1;4	PtPT4			15
P.trichocarpa	POPtr;Pht1;5	PtPT5			15
P.trichocarpa	POPtr;Pht1;6	PtPT6			15
P.trichocarpa	POPtr;Pht1;7	PtPT7			15
P.trichocarpa	POPtr;Pht1;8	PtPT8			15
P.trichocarpa	POPtr;Pht1;9	PtPT9	EM +		15
P.trichocarpa	POPtr;Pht1;10	PtPT10	AM +		15
P.trichocarpa	POPtr;Pht1;11	PtPT11	AM -		15
P.trichocarpa	POPtr;Pht1;12	PtPT12	EM +		15

Oryza sativa	ORYsa;Pht1;1	OSPT1	AM -		16
O. sativa	ORYsa;Pht1;2	OSPT2	AM -		16
O. sativa	ORYsa;Pht1;3	OSPT3	AM -		16
O. sativa	ORYsa;Pht1;4	OSPT4			16
O. sativa	ORYsa;Pht1;5	OSPT5			16
O. sativa	ORYsa;Pht1;6	OSPT6	AM -		16
O. sativa	ORYsa;Pht1;7	OSPT7			16
O. sativa	ORYsa;Pht1;8	OSPT8			16
O. sativa	ORYsa;Pht1;9	OSPT9	AM -		16
O. sativa	ORYsa;Pht1;10	OSPT10	AM -		16
O. sativa	ORYsa;Pht1;11	OSPT11	AM S		16, 17
O. sativa	ORYsa;Pht1;12	OSPT12			16
O. sativa	ORYsa;Pht1;13	OSPT13	AM +		16
Hordeum vulgare	HORvu;Pht1;1	HvPT1	AM -	9	18, 19
H. vulgare	HORvu;Pht1;2	HvPT2	AM -		18, 19
H. vulgare	HORvu;Pht1;3	HvPT3			18, 19
H. vulgare	HORvu;Pht1;4	HvPT4			19
H. vulgare	HORvu;Pht1;5	HvPT5			19
H. vulgare	HORvu;Pht1;6	HvPt6		385	19
H. vulgare	HORvu;Pht1;7	HvPT7			19
H. vulgare	HORvu;Pht1;8	HvPT8	AM +		19
Triticum aestivum	TRIae;Pht1;myc	TaPTmyc	AM S		19
Zea mays	ZEAma;Pht1;1	ZmPT2	AM -		20, 21
Z. mays	ZEAma;Pht1;2	ZmPT1			21
Z. mays	ZEAma;Pht1;3	ZmPT3			21
Z. mays	ZEAma;Pht1;4	ZmPT4	AM -		20, 21
Z. mays	ZEAma;Pht1;5	ZmPT5			21
Z. mays	ZEAma;Pht1;6	ZmPT6	AM +		19, 21

a) Name of plant P_i transporters according to the official nomenclature (Karandashov and Bucher 2005). **b)** Short name of P_i transporters as used in this thesis. **c)** Expression pattern of plant P_i transporters in response to AM symbiosis: mycorrhiza specific (AM S), up-regulated (AM+) or down-regulated (AM-). **d)** The method used to determine the apparent Km were heterologous expression in yeast or rice cell culture. **e)** References: 1: (Bunya et al. 1991); 2: (Harrison and Vanbuuren 1995); 3: (Maldonado-Mendoza et al. 2001); 4: (Benedetto et al. 2005); 5: (Liu et al. 1998a); 6: (Nagy et al. 2005); 7: (Leggewie et al. 1997); 8: (Rausch et al. 2001); 9: (Karandashov et al. 2004); 10: (Liu et al. 1998b); 11:(Harrison et al. 2002); 12: (Maeda et al. 2006); 13: (Tamura et al. 2012); 14: (Wu et al. 2011); 15: (Loth-Pereda et al. 2011); 16: (Paszkowski et al. 2002); 17: (Guimil et al. 2005); 18: (Rae et al. 2003); 20: (Glassop et al. 2005); 20: (Wright et al. 2005); 21:(Nagy et al. 2006). Table modified after(Javot et al. 2007b).

A2. AM-fungal phosphate transporters

Inorganic phosphate transporters of *Glomus intraradices* and *G. mosseae* expressed in flax and sorghum roots. Plants were part of the experiment described in chapter 2.

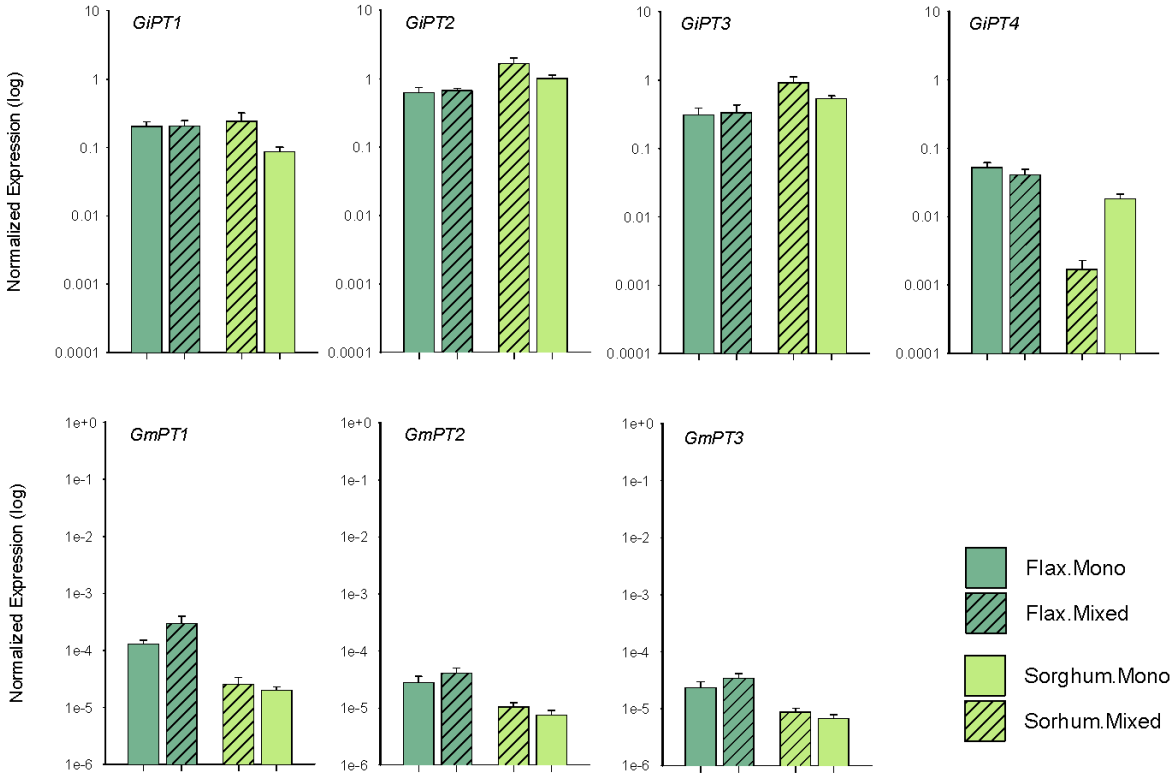


Figure A2.1 Quantification by qRT-PCR analysis of transcript levels of inorganic phosphate transporters of *Glomus intraradices* (Gi) an *Glomus mosseae* (Gm) in flax and sorghum roots. Plants were either grown as monoculture (Mono) or as mixed culture (Mixed). Transcript levels were normalized against glutamin-synthase in *G. intraradices*, and against ribosomal 35S RNA in *G. mosseae*. Values are means of four replicates, error bars represent SEM.

A3. Experiment 07

Additional data on the experiment 07, which is presented in Chapter 2 and 5. The set-up of experiments 07 is described in detail in chapter 2.

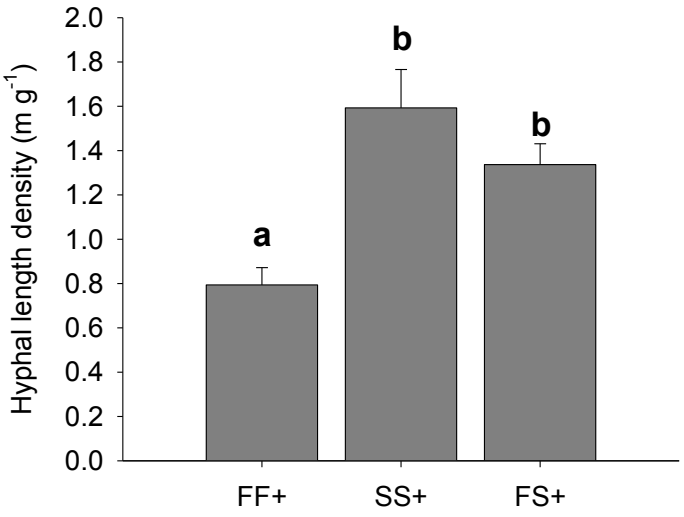


Figure A3.1 Means (±SE; n=4) of hyphal length density background corrected with the corresponding control treatments in hyphal compartments at harvest (12 weeks). The plants used were flax (F) and sorghum (S), grown either as a pair of conspecific plants (F:F, S:S) as a model of monoculture, or in combination (F:S) as a model of a mixed culture. Different letters above bars indicate a significant difference (P≤ 0.05), according to LSD test.

A4. Experiment 08

Data on the experiment 08, which is not presented in the main text. The set-up of experiments 08 is described in detail in chapter 2.

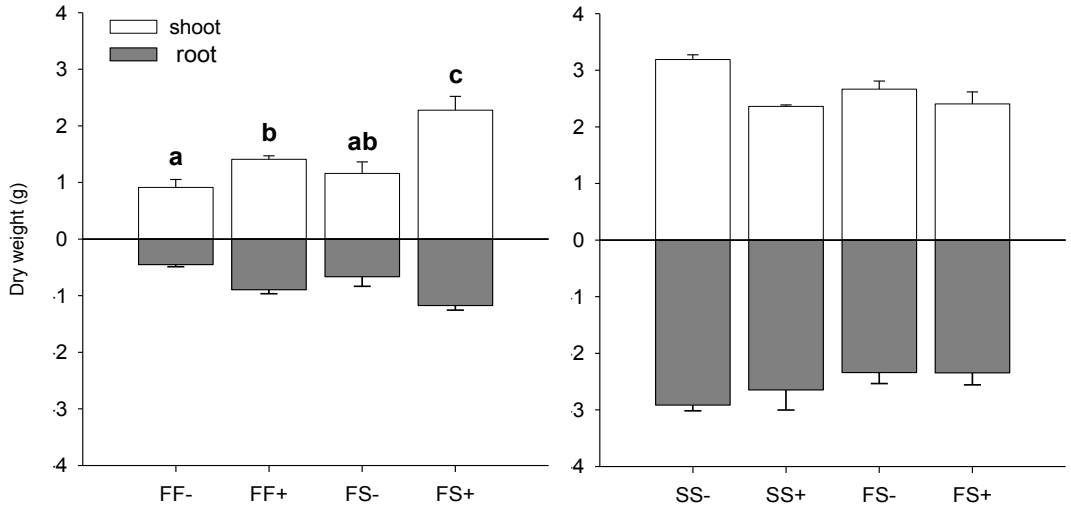


Figure A4.1 Mean (±SE, N=4) shoot (above x-axis) and root (positive values below x- axis) dry weights per plant at harvest (12 weeks). The plants used were flax (F) and sorghum (S), grown either as a pair of conspecific plants (F:F, S:S) as a model of monoculture, or in combination (F:S) as a model of a mixed culture. Mean comparison are treated separately for both plant species. Letters above bars indicate a significant difference (P≤0.05) of total dry weight among treatments, according to LSD test.

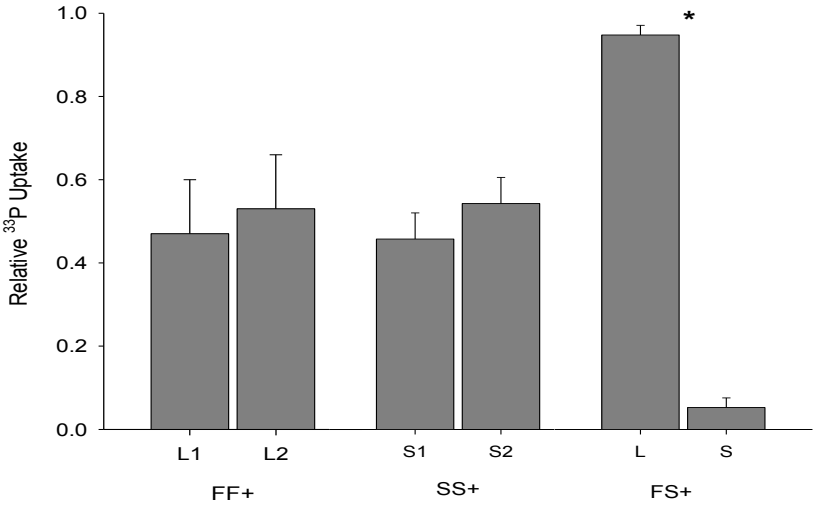


Figure A4.2 Mean (±SE) relative ^{33}P uptake via AMF hyphae in monoculture or in the competition between Flax (F) and sorghum (S). The plants were grown either as a pair of conspecific plants (F:F, S:S) as a model of monoculture, or in combination (F:S) as a model of a mixed culture. Relative uptake was calculated by dividing ^{33}P uptake of plant individuals by the total uptake per compartment. Mean comparison are treated separately for all treatments. Stars above bars indicate a significant difference (P≤ 0.05), according to One-Way ANOVA.

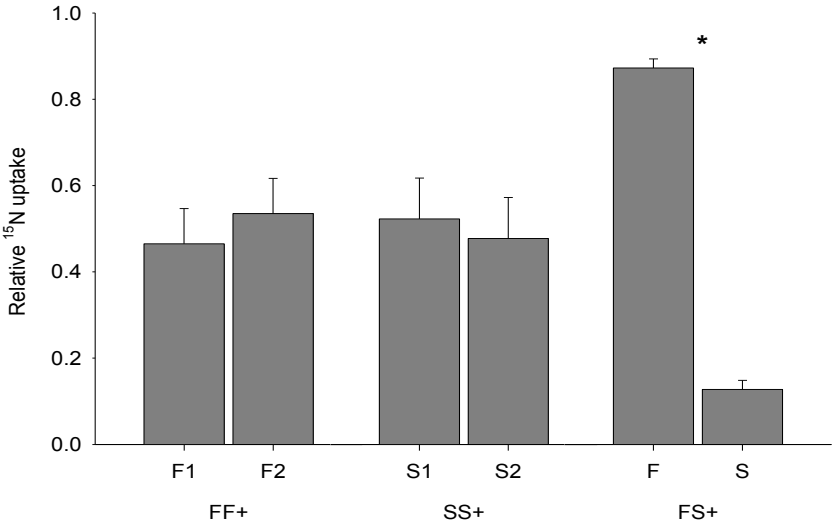
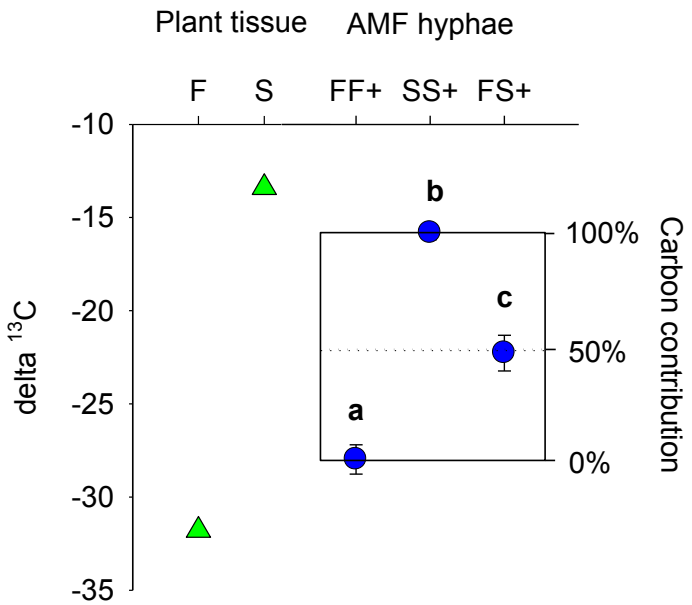


Figure A4.3 Mean (±SE) relative ^{15}N uptake in monoculture or in the competition between Flax and Sorghum. The plants used were grown either as a pair of conspecific plants (F:F, S:S) as a model of monoculture, or in combination (F:S) as a model of a mixed culture. Relative uptake was calculated by dividing ^{15}N uptake of plant individuals by the total uptake per compartment. Mean comparison are treated separately for all treatments. Stars above bars indicate a significant difference (P≤ 0.05, according to One-Way ANOVA.

Figure A4.4 Means (±SE) of δ ^{13}C values of plant tissue (green triangles) and of extracted hyphae of the hyphal compartments (blue circles) at harvest (12 weeks). The plants used were flax (F) and sorghum (S), grown either as a pair of conspecific plants (F:F, S:S) as a model of monoculture, or in combination (F:S) as a model of a mixed culture. Carbon contribution of sorghum to the hyphal network is shown in the box. Letters above bars indicate a significant difference (P≤0.05) of total dry weight among treatments, according to LSD test.



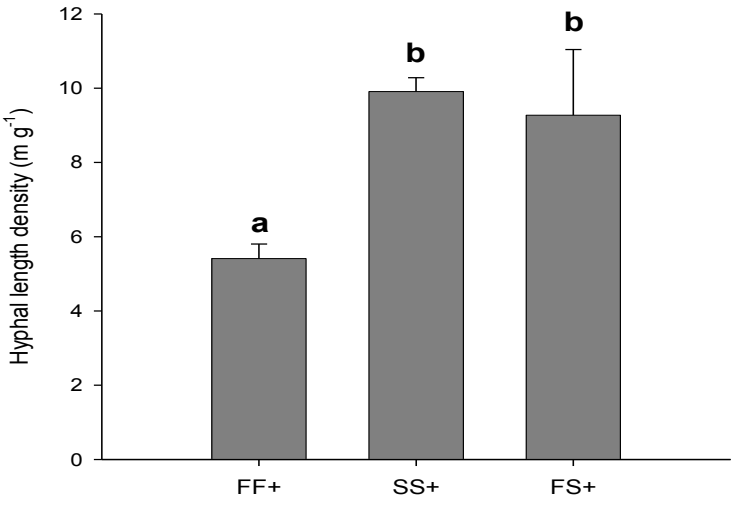


Figure A4.5 Means (±SE; n=4) of hyphal length density background corrected with the corresponding control treatment in hyphal compartments at harvest (12weeks). The plants used were flax (F) and sorghum (S), grown either as a pair of conspecific plants (F:F, S:S) as a model of monoculture, or in combination (F:S) as a model of a mixed culture. Different letters above bars indicate a significant difference (P≤ 0.05), according to LSD test.

A5. Experiment 09

Additional data on the experiment 09, which is presented in Chapter 5. The experiment 09 was conducted in the greenhouse with a slightly different compartment system that had no hyphal-label compartment (Figure 2.1). Additionally, the growth period was extended to 4.5 month to await sufficient spore production.

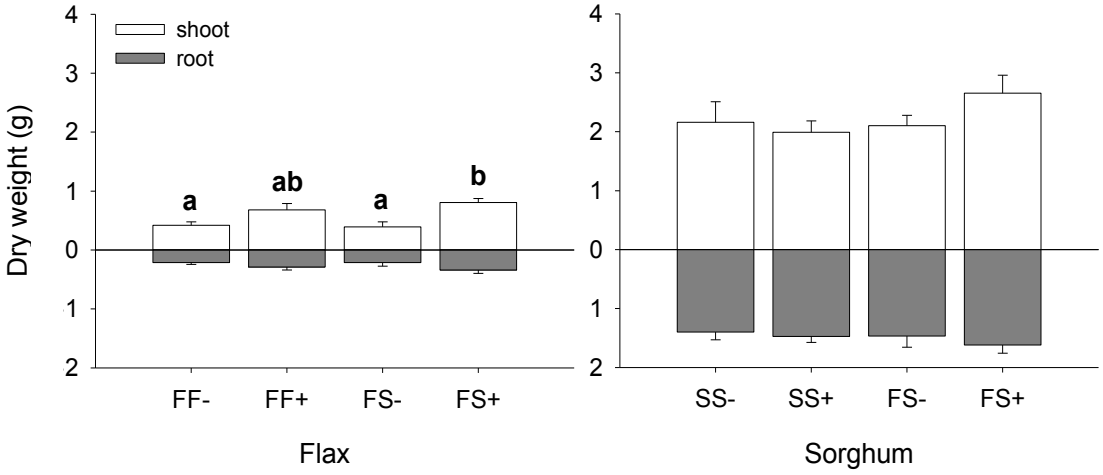
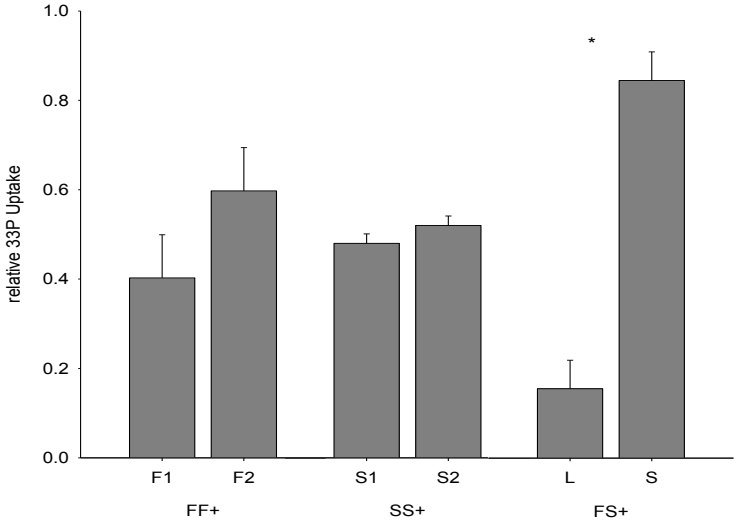


Figure A5.1 Mean (±SE) shoot (above x-axis) and root (positive values below x- axis) dry weights per plant at harvest (21 weeks). The plants used were flax (F) and sorghum (S), grown either as a pair of conspecific plants (F:F, S:S) as a model of monoculture, or in combination (F:S) as a model of a mixed culture. Mean comparison are treated separately for both plant species. Letters above bars indicate a significant difference (P≤0.05) of total dry weight among treatments, according to LSD test.

Figure A5.2 Mean (±SE) relative ^{33}P uptake via AMF hyphae in monoculture or in the competition between. Flax (F) and Sorghum. (S). The plants were grown either as a pair of conspecific plants (F:F, S:S) as a model of monoculture, or in combination (F:S) as a model of a mixed culture. Relative uptake was calculated by dividing ^{33}P uptake of plant individuals by the total uptake per compartment. Mean comparison are treated separately for all treatments. Stars above bars indicate a significant difference (P≤ 0.05), according to One-Way ANOVA.



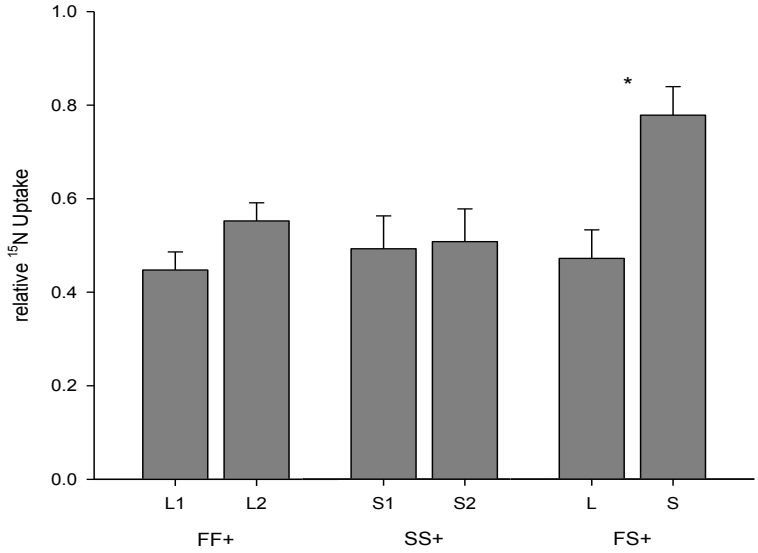


Figure A5.3 Mean (±SE) relative 15N uptake via AMF hyphae in monoculture or in the competition between. Flax (F) and Sorghum. (S). The plants were grown either as a pair of conspecific plants (F:F, S:S) as a model of monoculture, or in combination (F:S) as a model of a mixed culture. Relative uptake was calculated by dividing ^{33}P uptake of plant individuals by the total uptake per compartment. Mean com-parison are treated separately for all treatments. Stars above bars indicate a significant difference (P≤ 0.05), according to One-Way ANOVA.

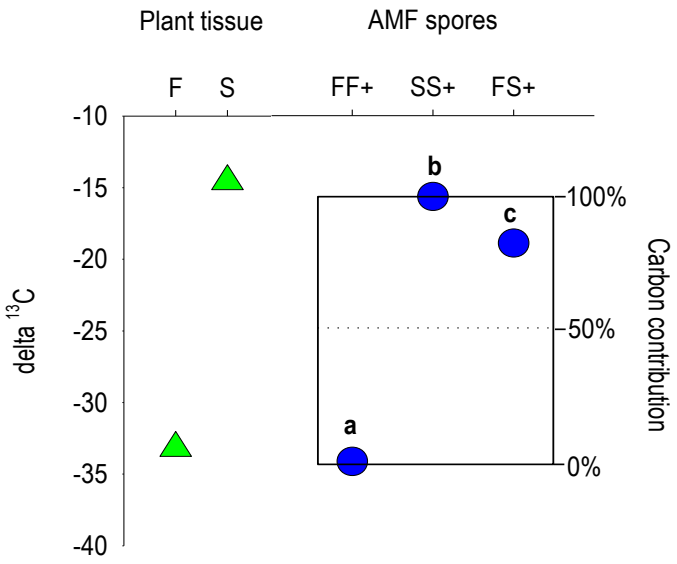


Figure A5.4 Means (±SE) of δ ^{13}C values of plant tissue (green triangles) and of extracted spores of hyphal compartments (blue circles) at harvest (21 weeks). The plants used were flax (F) and sorghum (S), grown either as a pair of conspecific plants (F:F, S:S) as a model of monoculture, or in combination (F:S) as a model of a mixed culture. Carbon contribution of sorghum to the hyphal network is shown in the box. Letters above bars indicate a significant difference (P≤0.05) of total dry weight among treatments, according to LSD test.

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9 CURRICULUM VITEAE

PERSONAL

Name	Florian Walder
Date of birth	29. November 1981
Nationality	Swiss

EDUCATION

04.2008 – 06.2012 **PhD in Botany,** Botanical Institute, University of Basel
PhD-Thesis: *Trade of carbon and soil nutrients between plants and fungi within the common arbuscular mycorrhizal network* (Grade: Magna cum laude)

- Development and use of assays to measure the carbon isotopic signature of arbuscular mycorrhizal fungi in field and greenhouse samples
- Conductance of functional experiments to track carbon and nutrient flows between plants and arbuscular mycorrhizal fungi in different culture systems
- Development and use of a gene expression assay for phosphate transporters of sorghum and flax

10.2005 – 04.2007 **Master of Science in Plant Science,** Botanical Institute, University of Basel
Master-Thesis: *Return of investments into a common mycorrhizal network for two different plant species* (Grade: 5.5)

- Conductance of functional experiments to investigate the role of arbuscular mycorrhizal fungi in mono- and mixed culture systems

10.2002 – 06.2005 **Bachelor of Science in Biology**, University of Basel

12.2001 **Matura** Typus E, Gymnasium Liestal

SCIENTIFIC WORK EXPERIENCE

04.2008 – 06.2012 **Botanical Institute**, University of Basel
Assistant (during the PhD)

- Support of Master and PhD students in planning and conductance of functional experiments
- Participation in teaching the courses “Blockkurs Planzenphysiologie” and “Pflanzenphysiologisches Praktikum”

06.2007 – 02.2008 **Syngenta Crop Protection**, Stein
Post-Graduate research associate in the “Resistance” group

- Genotyping of a *Phytophthora infestans* population by the use of microsatellites

WORK EXPERIENCE

11.2005 – 04.2007	**Rebisto GmbH**, Gelterkinden Tutor in natural sciences
08.2005 – 09.2005	**Gartenbau Hefel**, Roderdorf Gardener

LANGUAGE SKILLS

German	Mother tongue
English	Fluent in spoken and written
French	Elementary

COMPUTER SKILLS

Operating systems	Windows, OSX
Software	SPSS, Sigma Plot, EndNote, Mega

CONGRESSES & COURSES

07.2012	**7th International Symbiosis Society Congress**, Krakow, Poland *Oral presentation*: "Mycorrhizal networks are shared by the plants under unequal terms of trade"
09.2011	**12th European Ecological Federation Congress**, Avila, Spain *Oral presentation*: "Carbon investment and nutrient return relations of two plants sharing a hyphal network of arbuscular mycorrhizal fungi"
03.2011	**Indo-Swiss Collaboration in Biotechnology International Symposium**, New Delhi, India *Poster presentation*: "Uneven return on investments of two plants interconnected by a common hyphal network of arbuscular mycorrhizal fungi: a lesson for inter-cropping"
10.2010	**Summer School on Stable Isotopes in Grassland Science**, Technische Universität München, Munich, Germany
06.2010	**Swiss Plant Science Web-Summer School**: The global food crisis: how can plant science contribute?, Mürren, Switzerland *Poster presentation*: "Uneven return on investments of two plants joined by a common hyphal network of arbuscular mycorrhizal fungi "
08.2009	**6th International Symbiosis Society Congress**, Madison, USA *Poster presentation*: "Uneven return on investments of two plants joined by a common hyphal network of arbuscular mycorrhizal fungi"

AWARDS

2011	**Poster award**, Indo-Swiss Collaboration in Biotechnology International Symposium, New Delhi, India
2010	**Poster award**, Swiss Plant Science Web-Summer School: The global food crisis: how can plant science contribute?, Mürren, Switzerland

PUBLICATIONS

In preparation	"**Expression of phosphate transporter genes in Sorghum and flax: developmental aspects and regulation in common mycorrhizal networks**". Walder F.*, Courty P.E.*, Koegel S., Wiemken A. & Boller T. - New Phytologist
2013	"**Tracking the carbon source of arbuscular mycorrhizal fungi using natural abundance of the carbon isotope ^{13}C**", Walder F., Niemann N., Lehmann M.F., Boller T., Wiemken A. & Courty P.E. - Soil Biology & Biochemistry 58:341-344
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2011	"**Changes of genotype, sensitivity and aggressiveness in *Phytophthora infestans* isolates collected in European countries in 1997, 2006 and 2007**", Gisi U., Walder F., Resheat-Eini Z., Edel D. & Sierotzki H. – Journal of Phytopathology 159:223-232
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