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Cooperation and Punishment in the Arbuscular Mycorrhizal Symbiosis: Implications for Resource Exchange & Biological Market Dynamics

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COOPERATION AND PUNISHMENT IN THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS: IMPLICATIONS FOR RESOURCE EXCHANGE & BIOLOGICAL MARKET DYNAMICS

BY

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Biological Sciences degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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To God be the glory

Dedicated to Mensah family

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CONTENTS

ABBREVIATIONS

AM: Arbuscular mycorrhiza(l)

Arg: Arginine

C: Carbon

CMN: Common mycorrhizal network

DP: Direct plant pathway

ERM: Extraradical mycelium

IRM: Intraradical mycelium

N: Nitrogen

NH_4^+: Ammonium

NM: Non-mycorrhizal

NO_3^-: Nitrate

MP: Mycorrhizal pathway

P: Phosphate

P_i: Inorganic phosphate

Poly-P: Polyphosphate

qPCR: Real time quantitative PCR

SIP: Stable isotope probing

ABSTRACT

COOPERATION AND PUNISHMENT IN THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS: IMPLICATIONS FOR RESOURCE EXCHANGE & BIOLOGICAL MARKET DYNAMICS

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The arbuscular mycorrhizal (AM) symbiosis is arguably the world's most abundant and important mutualism, and brings together the roots of the majority of land plants and AM fungi to great mutual advantage. The AM symbiosis can increase the uptake of nutrients, such as phosphorus (P) and nitrogen (N), and improves the abiotic and biotic stress resistance of the host plant. AM fungi have the potential to act as biofertilizers and bioprotectors in sustainable agriculture. However, despite its significance, the mechanisms that control the resource exchange between both partners in the arbuscular mycorrhizal symbiosis are largely unknown. The main aim of this research project is to better understand the physiological mechanisms that control the cost to benefit ratios in the AM symbiosis, and to investigate how cooperation between partners is stabilized in the AM symbiosis on a cellular, whole plant and whole plant community level. This knowledge about AM interactions could help farmers to increase crop productivity under conditions that will very likely threaten food production in the future, e.g. drought by climate change, and the need to reduce fertilizer inputs.

The research project addresses the following research gaps:

1. How is cooperative behavior between symbionts enforced?
2. Is the fungal partner able to distinguish cooperative partners and to allocate resources accordingly?
3. Is plant growth benefit correlated to the P and N metabolism of the AM fungus?
4. Are all AM fungi equally beneficial?
5. Is carbon a trigger that stimulates P and N transport in common mycelia networks?

We addressed these gaps in the AM symbiosis using *in vitro* root organ cultures and whole plant systems at the physiological and molecular level. The results indicate that plants reward better fungal partners with more carbohydrates while in return; fungal partners enforce cooperation by providing more nutrients to plants that provide more carbohydrates. This reciprocal reward system is analogous to a market economy, where trade is favored with partners offering the best rate of exchange. Our results also demonstrate that fungi are able to distinguish among host plants interconnected by common mycorrhizal networks (CMN) that differ in the benefit they provide for the CMN and that AM fungi allocate P and N to the host plants within their CMN that are able to provide more carbon. Plant growth benefit was highly correlated to the efficiency with which AM fungi were able to take up N, P and to the capability of the AM fungus to store P. Overall, our results demonstrate our hypotheses that biological market dynamics theory regulate the resource exchange and the evolutionary stability in the AM symbiosis.

CHAPTER 1: LITERATURE REVIEW

1.1 ARBUSCULAR MYCORRHIZAL SYBIOSIS

One of the world's greatest agricultural problems that we face in the future will be food production. With the increase in the world's population, required production increases can only be met with the application of artificial fertilizer in agricultural production. The production of crops is directly correlated to the accessibility of nitrogen (N) and phosphorus (P) and the costs of fertilizers have increased in recent years. In the U.S. alone, farmers spend $24.8 billion for fertilizer, lime and soil conditioners in 2012 averaging $25,164 per farm according to the U.S Department of Agriculture (2013). But fertilizers are not only costly, but also damage the environment. The extensive use of N fertilizer in U.S. corn production alone is responsible for 30% of the non-renewable energy consumption and for 70% of the greenhouse gas emissions in corn production (Kim & Dale, 2008). The use of P fertilizers will even become more critical because phosphate rock that is used for the production of P fertilizers will likely be depleted in about 50-100 years (Vance *et al.*, 2003). The use of these fertilizers is responsible for a number of environmental costs in agricultural production, including the degradation of soil and water quality, eutrophication of marine ecosystems, photochemical smog and increasing concentrations of greenhouse gases.

The 450-million-year-old arbuscular mycorrhizal (AM) symbiosis is arguably the most important mutualistic symbiosis on Earth, and plays a key role in the nutrient supply and abiotic and biotic stress resistance of the majority of land plants. The symbiosis

brings together the roots of over 80 % of land plant species (such as wheat, corn, soybean and rice)(Wang & Qiu, 2006) and fungi of the phylum *Glomeromycota (Schubler et al., 2001)* to great mutual advantage. The AM symbiosis increases the resistance of plants against abiotic (drought, heavy metals) and biotic (pathogens) stresses (Smith & Read, 2010). Fossil records suggest that the AM symbiosis facilitated the transition of plants from aquatic to terrestrial environments over 450 million years ago (Heckman *et al.*, 2001) and that the symbiosis played a critical role during plant evolution. The nutrient exchange mechanisms involved between plant and AM fungi are controlled by molecular symbiotic toolkits in the colonization and nutrient exchange (Delaux *et al.*, 2013).

The role of these ubiquitous soil fungi for plant productivity and health has prompted agronomic interest in these interactions with regard to a potential use as 'biofertilizers' in sustainable agriculture. However, despite the significance of this symbiosis, the mechanisms that control the resource exchange between both partners in this symbiosis are largely unknown. AM fungi are obligate biotrophs, that completely depend on their host plant for their survival and reproduction and the roots secrete strigolactones that stimulate the germination of the AM fungal spores (Akiyama & Hayashi, 2006). AM fungi form hyphopodia on the surface of the root and then penetrate through the root cells into the root cortex (Fig. 1). The fungal hyphae enter the apoplast penetrating into the cortical cells of the roots. The fungus forms highly branched structures known as arbuscules within the root cortical cells, which serve as the active site for the bi-directional nutrient exchange in the AM symbiosis. Most fungi are also able to form intercellular vesicles that serve as fungal nutrient storage compartments within the root apoplast. The extraradical mycelium (ERM) of the fungus acts as an extension of the root

system and takes up P, N, sulfur and trace elements from the soil, and delivers these nutrients via the intraradical mycelium (IRM) to the plant (Jakobsen *et al.*, 1992; Hawkins *et al.*, 2000; Allen & Shachar-Hill, 2009; Smith & Smith, 2011). In exchange, the plant allocates up to 20 % of its photosynthetically fixed carbon to the fungus (Wright *et al.*, 1998), this is equivalent to about 5 billion tons of carbon per year (Bago *et al.*, 2003) that is sequestered into the soil and that the fungus uses it to maintain its symbiotic structures and cell metabolism.

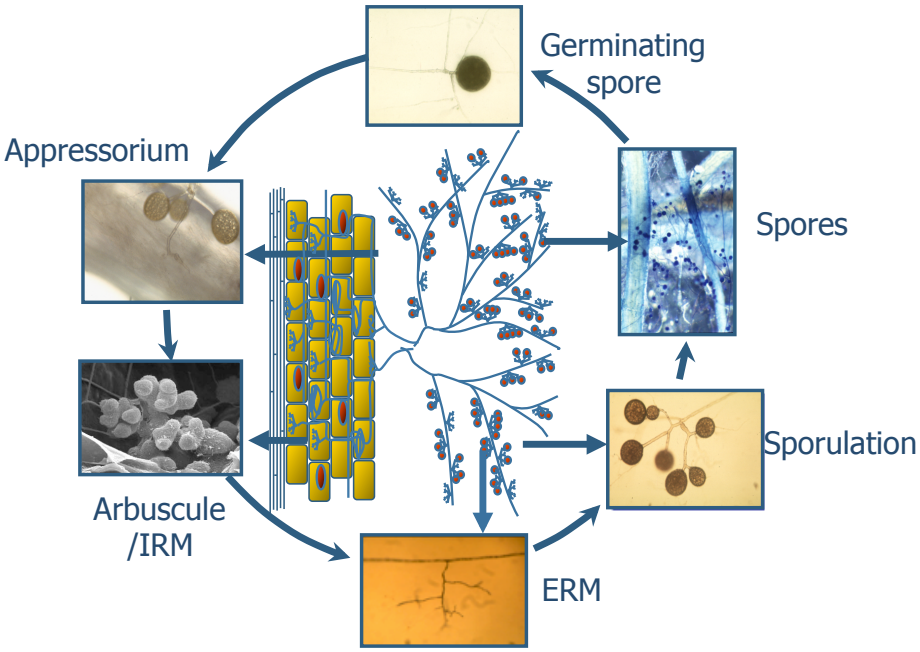

Fig. 1. Life cycle of an AM fungus and the different steps during AM development.

Plants can take up nutrients from the soil via the plant or mycorrhizal pathway or a combination of both (Fig. 2). The direct uptake of nutrients via the plant root epidermis is often limited by the slow movement of some nutrients such as phosphate (P_i) and the development of depletion zones around the roots that further limit P_i uptakes (Schachtman *et al.*, 1998). However, via the mycorrhizal pathway nutrients are taken up through the ERM of the AM fungus and taken up by the plant from the mycorrhizal interface. It has been estimated that the ERM can reach 100m of hyphae per cubic cm of soil (Parniske, 2008), and the ERM transfers nutrients to the IRM and transfers the nutrient across the interfacial apoplast to the plant (Bücking *et al.*, 2012).

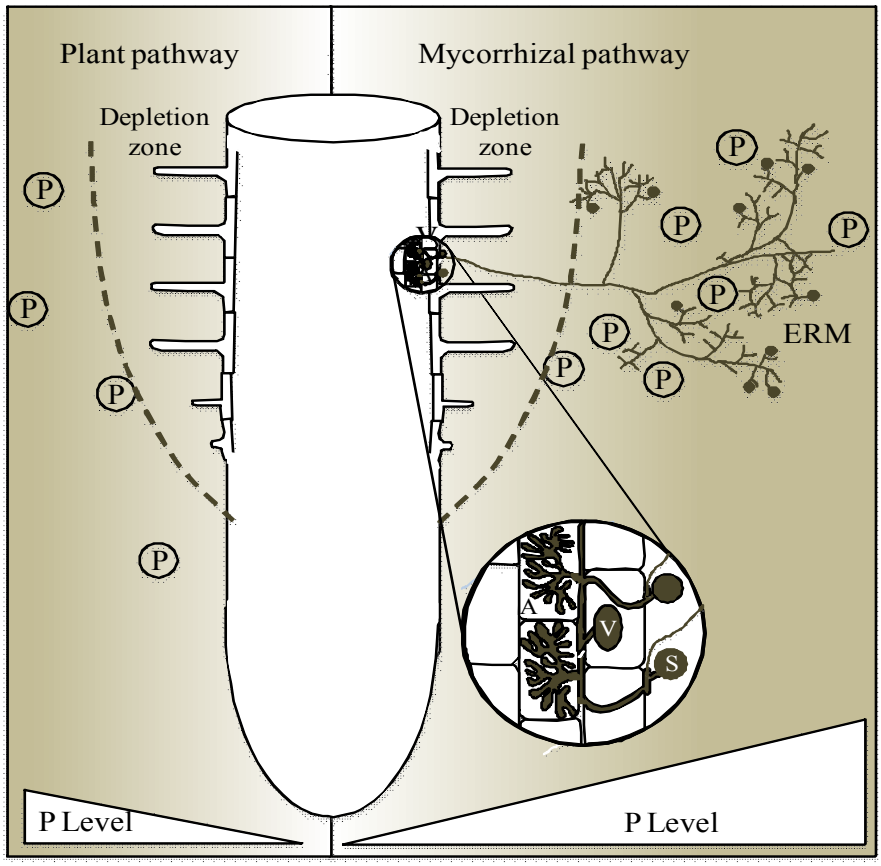

Fig. 2. Plant and mycorrhizal P uptake pathway. The AM fungus forms the extraradical mycelium (ERM) and extends the roots beyond the depletion zone and is able to take up nutrients. Within the host root, the fungus forms the intraradical mycelium (IRM) and highly branched arbuscules (A) where the exchange of nutrients takes place. Vesicles (V) and spores (S) are also formed by the AM fungus (Bücking *et al.*, 2012).

Mycorrhizal plants change their nutrient acquisition strategies. Multiple studies have indicated that the host plant may acquire the majority of its phosphate from the mycorrhizal fungus (Smith *et al.*, 2009). This has been demonstrated in *Medicago truncatula* (Grunwald *et al.*, 2009), where six known phosphate transporters (*MtPt 1-6*) were used as example. Five of the phosphate transporters (*MtPt 1-3,5,6*) were expressed at the plant root epidermis and the interface with the soil solution, while *MtPt4* transporter was induced only in the mycorrhizal plants and localized in arbusculated root cortical cells (Harrison *et al.*, 2002). The AM inducible *MtPt4* is up-regulated in the colonized roots while the other phosphate transporters are down-regulated (Grunwald *et al.*, 2009).

Studies have shown that both plants and AM fungi can interact with multiple partners and form common mycorrhizal network (CMN) (Fellbaum *et al.*, 2014). A host plant can interact with multiple AM fungi at the same time (Jansa *et al.*, 2008) while one fungal individuum can colonize multiple hosts of the same or of different species that provide different growth benefits (Giovannetti *et al.*, 2004; Mensah *et al.*, 2015). The interconnection of the CMN improves many-to-many interaction in host plants (Simard & Durall, 2004), soil composition (Hodge *et al.*, 2001) and soil microbe composition (Hodge, 2000). Recent studies support inter-plant communication in tomato plants under attack send herbivore-induced dense signals through CMN to other plants to alert them to prime their defense systems too (Barto *et al.,* 2012) before caterpillar or aphid attack (Babikova *et al.,* 2013; Song *et al.,* 2014). The interaction between host plants and AM fungi in the CMN functions as a biological investment (Walder *et al.*, 2012) between both partners, where partners seek to get access to resources at the best exchange rate.

1.2 PHOSPHATE NUTRITION

Inorganic phosphate (P_i) is known to be one of the major nutrients in the soil but has a low mobility rate in the soil solution (Smith & Smith, 2011). Therefore, AM fungi play an important role in the nutrient transfer of P for the plant. Inorganic phosphate (P_i) that is taken up by the ERM, and can replenish the metabolically active P_i pool in the hyphae that is used for the synthesis of phospholipids, DNA-, RNA- or protein-phosphates, or can be converted into long-chained or short-chained polyphosphates (poly-P) (Fig. 3). Poly-P are linear polymers of up to several hundred P_i residues linked by energy-rich phospho-anhydride bonds. Poly-P are rapidly synthesized in the hyphae of the ERM (Ezawa *et al.*, 2004) presumably by the poly-P polymerase/vacuolar transporter chaperone complex (VTC; (Tisserant *et al.*, 2012), and this poly-P accumulation is followed by a near-equivalent cation uptake by the fungal hyphae (Kikuchi *et al.*, 2014). Poly-P play an important role in the storage of P in the fungal hyphae, but also in the translocation of P from the ERM to the IRM (Hijikata *et al.*, 2010). In the IRM long-chained poly-P are broken down first into shorter chain lengths by a vacuolar endopolyphosphatase, followed by an exopolyphosphatase that hydrolyzes the terminal residues from the short-chain poly-P and releases P_i that can be transferred across the mycorrhizal interface to the host (Ezawa *et al.,* 2001; Tisserant *et al.*, 2012).

Considering the important role that poly-P play in P and N transport in the AM symbiosis, more knowledge about the poly-P metabolism and remobilization may contribute to a better understanding of the differences in the growth and nutritional benefits across by diverse fungal isolates. AM fungi differ in their poly-P metabolism (Boddington & Dodd, 1999) and the regulation of poly-P formation and/or remobilization

in the IRM provides the fungus with an instrument to regulate the P and N transport across the mycorrhizal interface (Bücking & Shachar‐Hill, 2005; Ohtomo & Saito, 2005; Takanishi *et al.*, 2009).

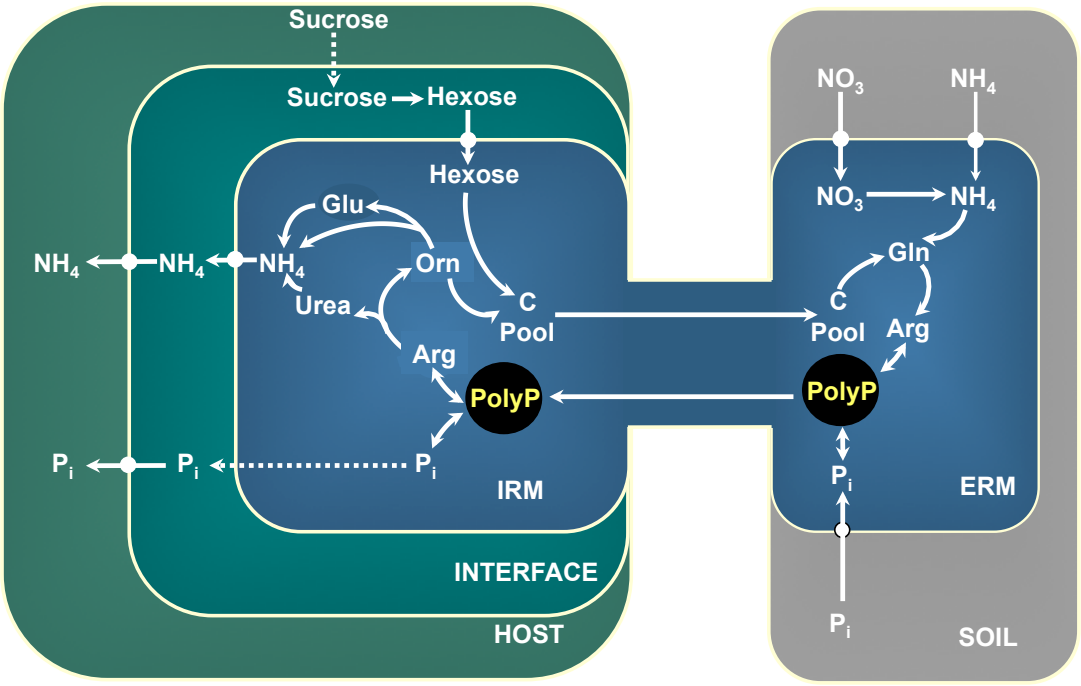

Fig. 3. The model shows the nutrient (N, P) transport from the extraradical mycelium (ERM) in the AM symbiosis. The ERM absorbs inorganic orthophosphate (P_i) from the soil. The P_i is transformed to long-chained polyphosphates. These long-chained polyphosphates are transferred to the intraradical mycelium (IRM) and broken down to short-chained polyphosphate. Likewise, nitrogen (N) is assimilated into arginine (Arg) through the anabolic arm of the urea cycle and transferred with polyp from the ERM to the IRM. P_i and NH_4^+ transporters play an important role in the uptake of the nutrients from the interfacial apoplast by the host. In exchange, sucrose is hydrolyzed in the interfacial apoplast to hexoses and taken up by the fungus.

1.3 NITROGEN NUTRITION

Nitrogen (N) is one of the most essential nutrients in the development of the plants (Botton & Chalot, 1999; Vitousek & Howarth, 1991)so N deficiency becomes a major limiting factor in plant productivity (Graham & Vance, 2000). Several works have demonstrated that N nutrition play an important role in the AM symbiosis (Smith & Smith, 2011; Hodge & Storer, 2015). The AM fungal species take up both NO_3^- and NH_4^+ from the soil (Fig. 3) (Johansen *et al.*, 1992, 1993; Tobar *et al.*, 1994; Bago *et al.*, 1996;Tanaka & Yano, 2005) and transfer N to their host plant (Hawkins *et al.*, 2000; Azcón *et al.*, 2001; Vázquez *et al.*, 2001). AM fungi can increase the uptake of organic N sources (Hawkins *et al.*, 2000) and the translocation by the fungus can represent a significant route of N uptake by the plant (Ames *et al.*, 1983; Johansen *et al.*, 1996). Depending on N availability and mobility this may represent an important benefit to the plant, but the mobility of N in soils has caused difficulty in assessing the importance of the fungal N transfer for plant nutrition (He *et al.*, 2003), and the contribution of AM fungi to the N nutrition of the host is still being questioned. However, there can be no reasonable doubt that AM fungi do take up N and transfer it to their hosts, and experiments in which the fungal extraradical mycelium had access to a completely separate compartment indicate that fungal uptake can account for a substantial part of total N uptake. For example, (Toussaint *et al.*, 2004) found that in an *in vitro* mycorrhiza where mycorrhizal roots had access to N both by direct uptake and via the mycorrhizal uptake at least 21 % of the total N uptake in AM roots came from the fungal extraradical mycelium; and Govindarajulu *et al.*, (2005) observed an even larger proportion (> 30 %).

1.4 COOPERATION AND PUNISHMENT IN THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS

The 450-million-year-old mutualism between plants and AM fungi is one of the most ancient, abundant, and ecologically important mutualisms on earth, because AM fungi play a key role for the productivity of 65% of all known land plant species, including numerous food and bioenergy crops. The mycorrhizal symbiosis is an ideal model system that can help to understand the evolution of punishment and cooperation in the symbiosis. Host and symbiont interact simultaneously with other individuals (Simard & Durall, 2004) and create highly dynamic and complex interactions (Selosse *et al.*, 2006). The symbiosis is energetically expensive, consuming between 5-20% of plant assimilates (Douds *et al.*, 2000), which explains why the hosts are under strong selection pressure to resist non-cooperators. The impact of different AM fungi on plant growth highly varies ranging from mutualistic to antagonistic (Klironomos, 2003; Egger & Hibbett, 2004; Jones & Smith, 2004), and is context –dependent (Fitter, 1991; Jones & Smith, 2004; Fitter, 2006). The AM symbiosis increases the resistance of plants against abiotic (drought, heavy metals) and biotic (pathogens) stresses (Smith & Read, 2008). Certain plants have adopted different structures to restore their nutrients back to the soil by forming mutualistic interactions with N-fixing bacteria and AM fungi. The AM fungi attacking large runner hyphae would enhance the fungus to colonize new host plants and obtain carbohydrates (Graham & Abbott, 2000; Hart & Reader, 2005). The plants are able to transfer C to the AM fungus and the increase in sporulation aid in the nutrient uptake to the host plant (Douds & Schenck, 1990). When the content of C is low for the fungus, less polyphosphate will be remobilized and the cytoplasmic concentration of

inorganic P in the IRM decreases and lowers P transport across the interface that results in a lower P availability for the host plant.

The distinction performance of some AM fungi are based on their environmental or molecular strategies. These fungi are able to distinguish host plants that differ in the benefit that they provide and allocate P accordingly to the host plants that are able to provide more carbon (Kiers *et al.*, 2011). Also, plants reward better fungal partners with more carbohydrates and in return; fungal partners enforce cooperation by providing more nutrients to plants that provide more carbohydrates. This reciprocal reward system is analogous to a market economy, where trade is favored with partners offering the best rate of exchange.

1.5 QUESTIONS, HYPOTHESES AND APPROACH

P nutrition has been shown to play an important role in the AM symbiosis and recent work has demonstrated that fungal partners are able to discriminate among host plants and preferentially allocate P and N (Bücking & Shachar‐Hill, 2005; Hammer *et al.*, 2011). Our main focus is to use cooperation in the AM symbiosis to understand the evolutionary context of enforced cooperation (West *et al.*, 2007) and to investigate how cooperation is stabilized in the AM symbiosis on a cellular, whole plant and whole plant community level. This would allow us to determine the physiological mechanisms that stabilize evolutionary cooperation as exerted by both partners in the AM symbiosis and the biological market that control cost to benefit ratios.

The following questions will be addressed to fill these gaps in our knowledge:

1. How is cooperative behavior between symbionts enforced?
2. Is the fungal partner able to distinguish cooperative partners and to allocate resources accordingly?
3. Is plant growth benefit correlated to the P and N metabolism of the AM fungus?
4. Are all AM fungi equally beneficial?
5. Is carbon a trigger that stimulates P and N transport in common mycelia networks?

The following chapters answer these questions with different experiments using root organ cultures and whole plant mycorrhizal systems. The experiments were carried out by different approaches to better understand how plant-microbe interactions drive the ecological processes and evolutionary trajectories of natural and agricultural ecosystems.

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CHAPTER 2: RECIPROCAL REWARDS STABILIZE COOPERATION IN THE MYCORRHIZAL SYMBIOSIS

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2.1 ABSTRACT

Plants and their arbuscular mycorrhizal fungal symbionts interact in complex underground networks involving multiple partners. This increases the potential for exploitation and defection by individuals, raising the question of how partners maintain a fair, two-way transfer of resources. We manipulated cooperation in plants and fungal partners to show that plants can detect, discriminate, and reward the best fungal partners with more carbohydrates. In turn, their fungal partners enforce cooperation by increasing nutrient transfer only to those roots providing more carbohydrates. On the basis of these observations we conclude that, unlike other mutualisms, the symbiont cannot be 'enslaved'. Rather, the mutualism is evolutionarily stable because control is bidirectional. Partners offering the best rate of exchange are rewarded.

2.2 INTRODUCTION

The symbiosis between plants and arbuscular mycorrhizal (AM) fungi is arguably the world's most prevalent mutualism. The vast majority of land plants form AM interactions, in which plants supply associated AM fungi with carbohydrates, essential for fungal survival and growth (Parniske, 2008) (Fig. S1). In exchange, AM fungi provide their host plants with mineral nutrients (e.g. phosphorus (P)) and other benefits such as protection against biotic (pathogens, herbivores) and abiotic stresses (e.g. drought) (Smith *et al.*, 2010). This partnership, which evolved long before mutualisms among insects or vertebrates (Leigh, 2010), is credited with driving the colonization of

land by plants, enabling massive global nutrient transfer and critical carbon sequestration (Bonfante & Genre, 2010; Smith *et al.*, 2010).

The selective forces maintaining cooperation between plants and AM fungi are unknown (Johnson *et al.*, 1997; Fitter, 2006; Kiers & Heijden, 2006; Bonfante & Genre, 2010; Leigh, 2010). Providing nutritional benefits can be metabolically costly, leading to the expectation that partners may defect from mutualistic duties (Kiers & Heijden, 2006; Douglas, 2008). If individual host plant and fungal symbiont interests are tightly aligned (Poulsen & Boomsma, 2005), fungal symbionts will increase their own fitness by helping plants grow (Frank, 1996), and vice versa. However, plants are typically colonized by multiple fungal strains (Vandenkoornhuyse *et al.*, 2007) and fungal 'individuals' can simultaneously interact with multiple host plants (Mikkelsen *et al.*, 2008) or species (Selosse *et al.*, 2006)(Fig. S1). This can select for 'cheaters' that exploit the benefits provided by others, while avoiding the costs of supplying resources (Douglas, 2008; Leigh, 2010). It is possible that plants have evolved mechanisms to enforce cooperation by fungi, analogous to the sanctions against uncooperative partners demonstrated in diverse mutualisms (Pellmyr & Huth, 1994; Kiers *et al.*, 2003; Goto *et al.*, 2010; Jandér & Herre, 2010). However, sanction mechanisms in other systems appear to rely on a single host interacting with, and controlling the fate of, multiple partners. In contrast, the AM symbiosis involves a complex series of many -to- many interactions with multiple fungal strains (Vandenkoornhuyse *et al.*, 2007) and multiple hosts (Selosse *et al.*, 2006), and so it is not clear whether sanctions could operate in the same way.

An alternative explanation for the stability of the plant-mycorrhizal mutualism is that both plants and fungi are able to detect variation in the resources supplied by their

partners, allowing them to adjust their own resource allocation accordingly. Such exchange of resources, in economic terms, represents a 'biological market', in which partners exchange commodities to their mutual benefit (Noë & Hammerstein, 1995; Schwartz & Hoeksema, 1998; Bshary & Noë, 2003; De Mazancourt & Schwartz, 2010). However, whilst mutualism market analogies have a strong theoretical basis (Schwartz & Hoeksema, 1998; Hoeksema & Kummel, 2003; Cowden & Peterson, 2009), plants may be unable to discriminate among intermingled fungal species on a fine enough scale to reward individual fungi (Bever *et al.*, 2009). Empirical tests have previously been constrained by our inability to track host resources into diverse AM assemblages, and difficulties in manipulating the cooperative behavior of both fungal and plant partners.

We resolve these constraints by allowing fungal genotypes that differ in their cooperative behavior to compete directly on a single root system. We used stable isotope probing to track and quantify plant resource allocation to individual fungal species (SIP, Fig. S2) (Vandenkoornhuyse *et al.*, 2007), and hence test for host discrimination against less cooperative partners. We also employ *in-vitro* root organ culture approaches (Pfeffer *et al.*, 1999) to manipulate cooperative behavior of both plant and fungal mutualists to examine patterns of reciprocal rewards in response to variable levels of cooperation.

(Material).

We used the model plant *Medicago truncatula* and three arbuscular mycorrhizal fungal species within the cosmopolitan sub-genus Glomus Ab (*Glomus intraradices*, *G. custos*, and *G. aggregatum*). These AM fungi exhibit either high or low levels of cooperation (symbiont quality), based on plant growth responses, costs of carbon per unit P transferred, and resource hoarding strategies, with the two less-cooperative species

directing more carbon resources either into storage vesicles (*G. aggregatum*) or spores (*G. custos*) compared to the cooperative species (Figs. S3, S4). We used closely-related species to avoid potential confounding factors attributed to differences in life history traits not linked to nutrient exchange (Powell *et al.*, 2009). We do not categorize our less-cooperative species as unequivocal 'cheats', noting that they may confer other benefits not measured here (Material).

We grew *Medicago* hosts with one, two (*G. intraradices* vs. *G. aggregatum*) or all three AM fungal species. We followed the C-flux through the plant to the fungal partners by tracking plant-assimilated C after 6 h in a ^{13}CO2 atmosphere (Vandenkoornhuyse *et al.*, 2007). We harvested the roots after 6, 12 and 24 h to follow the incorporation of host carbon into the RNA of the AM fungal community. We focused on RNA because it better reflects immediate C allocation patterns relative to DNA (Manefield *et al.*, 2002). Total RNA extractions were then subjected to ultracentrifugation to separate fractions based upon the level of ^{13}C incorporation. By quantifying ribosomal rRNA transcripts via quantitative PCR, we were able to track the relative C allocation to each of the AM fungal species (Fig. S2, S9, S10).

We found that more carbon was supplied to the more cooperative fungal species. In both the two-species and three-species experiments, the RNA of the cooperative fungus, *G. intraradices*, was significantly more enriched with host ^{13}C than the RNA of both less-cooperative species of the same genus (Fig. 1).

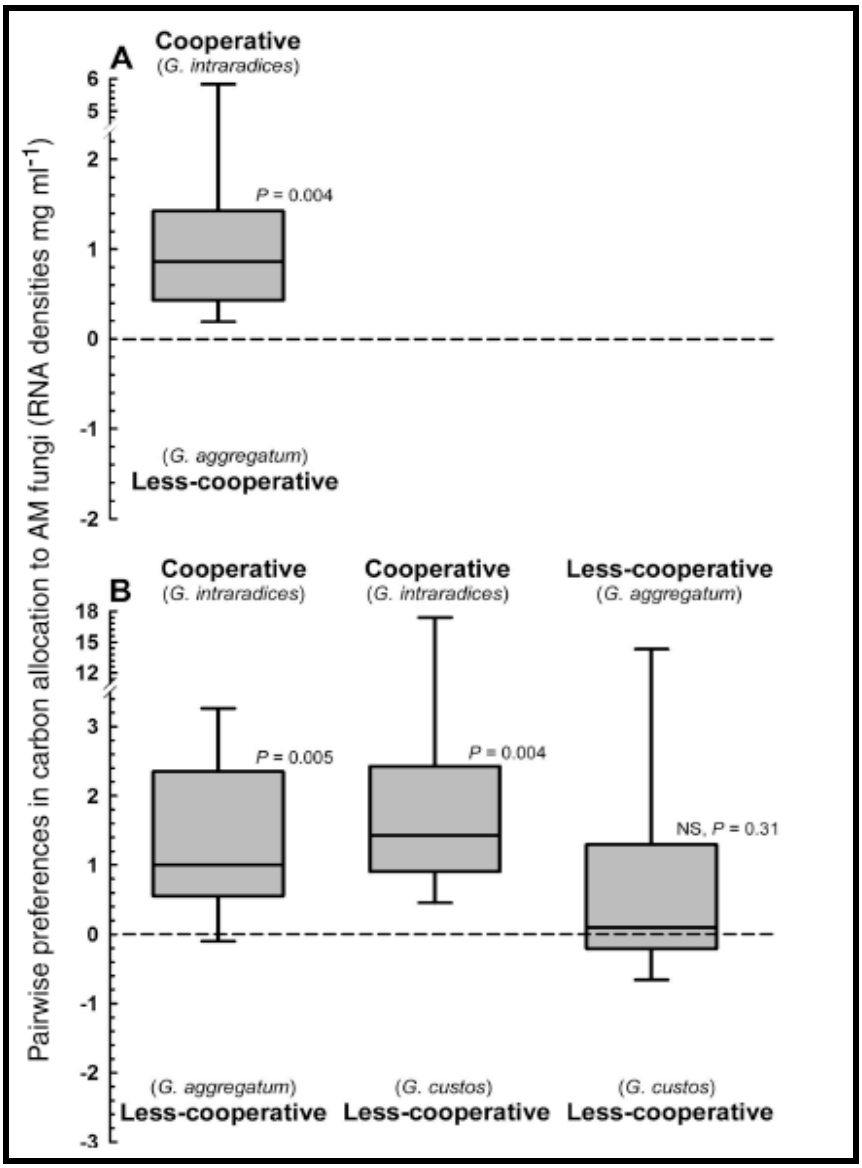

Fig. 1. Pair-wise comparisons of carbon allocation patterns to co-exisiting AM fungal species based on C-enrichment. Values above zero line indicate preferential allocation to species above the line. (**A**) More carbon was allocated to the cooperative species (*G. intraradices*) compared to the less cooperative species (*G. aggregatum*) in a two-species experiment. (**B**) When host plants were colonized with three AM fungal species, the RNA of the cooperative species (*G. intraradices*) was again significantly more enriched than that of the two less-cooperative species (*G. aggregatum* and *G. custos*). There was no significant difference in RNA enrichment between the two less-cooperative species. Data from all harvest times were pooled because no significant effect of time on RNA enrichment (Kruskal-Wallis, $P > 0.05$ for all three fungal species). Middle lines of box plots represent median values (n=11) with bars showing value ranges (minimum to maximum). *P*-values refer to nonparametric sign tests for differences of sample median from zero.

We reject the hypothesis that the less-cooperative species were simply incompatible partners because colonization in all single-species controls were above 80% (Fig. S4). Moreover, we found a significant effect of host preference on fungal abundance. *G. aggregatum* decreased by 36.7% ($F_{1,8} = 6.39$, $P = 0.035$) and *G. custos* by 85% ($F_{1,8} = 63.6$, $P < 0.001$) in communities where a high-quality partner was available (Fig. S5), suggesting either a shift in resource supply by the host to the more cooperative species or changes in competitive dynamics among the fungi (Material).

The extent to which cooperation can be effectively enforced depends upon the scale at which hosts discriminate against less-cooperative fungal symbionts. For plant hosts, this detection would have to occur at very fine spatial scales (e.g. ~cm or smaller), because genetically-distinct fungi can form closely intermingled networks within host root systems (Parniske, 2008). However, it has been argued that plants cannot discriminate among mixed fungi once colonization has been established (Bever *et al.*, 2009). Discrimination on the basis of fungal signaling is unlikely because there is no reason that fungi would have to signal honestly (Leigh, 2010)

To resolve this potential paradox, we tested whether fine-scale host discrimination occurs between fungal hyphae colonizing the same host root. We used an *in-vitro* triple split-plate system, with one mycorrhizal root compartment and two fungal compartments composed of the same fungal species, but varying in P supply. This allowed us to mimic cooperation or defection by fungal partners connected to the same host root, and track how this influences C allocation back to the fungus (Fig. 2 A, B). If hosts rely on nutrient transfer as a tool to discriminate between partners on the same root (Fitter, 2006; Kiers &

Heijden, 2006), we would predict higher C allocation to the hyphae with access to higher P resources.

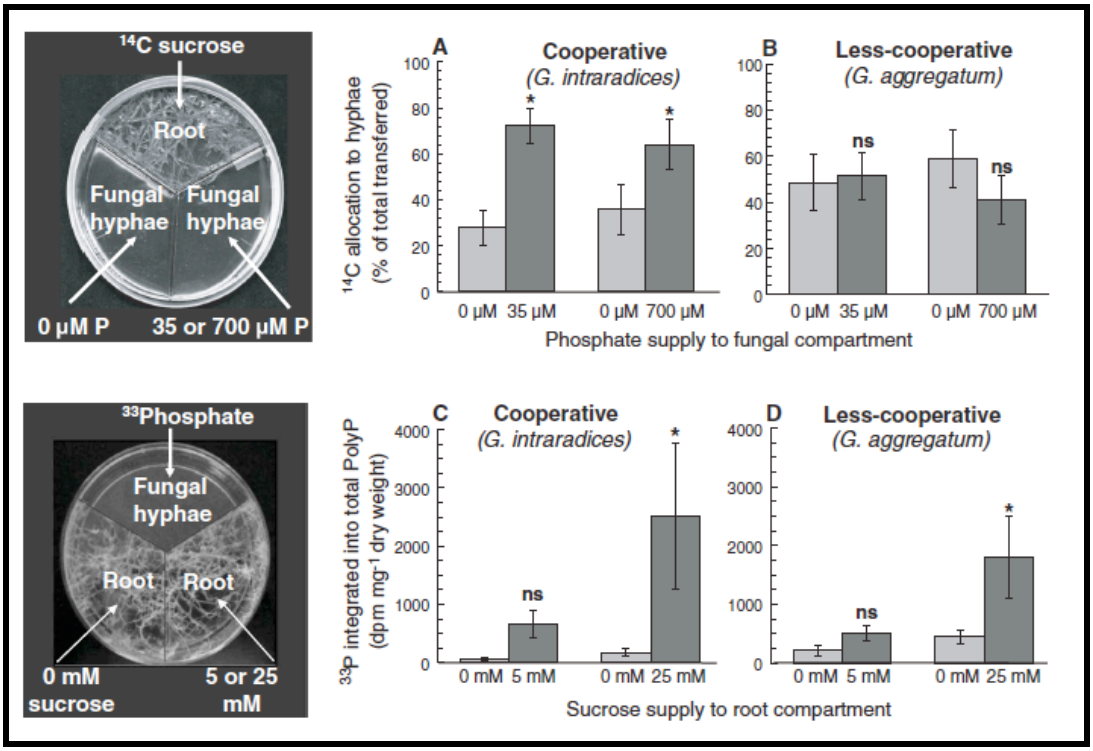

Fig. 2. Triple-plate experiments to mimic partner cooperation or defection. We found a significant effect of P-availability on C allocation patterns ($F_{3,20} = 5.29$, $P = 0.0075$), with preferential allocation of C to the fungal compartments with access to more P in (**A**) *G. intraradices,* but not in (**B**) *G. aggregatum*. In the reciprocal experiment, we found a significant effect of the C supply on P allocation patterns ($F_{7,58}$=7.298, P <0.0001) with a higher allocation of fungal P (measured as polyphosphate, PolyP) to root compartments with higher C in both (**C**) *G. intraradices* and (**D**) *G. aggregatum*. However, the less-cooperative species, *G. aggregatum*, remobilized a smaller percentage of its long-chained PolyP into short-chained PolyP, indicative of a hoarding strategy (figs. S6, S8). Asterisks show significant differences between treatment means (Student- Newmans- Keuls test, $P \leq 0.05$). Bars represent the means of 8-10 replicates ± 1 standard error.

We found that hosts rewarded fungal hyphae that were supplied with greater P resources. As predicted, four days after the addition of ^{14}C labeled sucrose to the root compartment, we found that significantly more C was transferred to the fungal hyphae with access to more P (Fig. 2A). In the cooperative species, *G. intraradices*, even small quantities of available P (e.g. 35 μM) resulted in a 10-fold increase in C allocation to the hyphae, relative to the hyphae with no access to P. We found no C allocation differences when hosts were colonized by the less-cooperative species, *G. aggregatum* (Fig. 2B).

Like their plant hosts, AM fungi interact with multiple partners in nature (Selosse *et al.*, 2006). Consequently, fungi may also enforce cooperation by rewarding increased C supply with greater P transfer. We used a reciprocal triple split-plate experimental design, with one fungal and two root compartments, to test whether the fungal partner would preferentially allocate P to the host providing more carbohydrates (Fig. 2 C,D). We found that the cooperative species transferred more P into roots with greater access to C resources (Fig. 2C), confirming it could discriminate among hosts differing in C supply. In contrast, the less-cooperative species, *G. aggregatum,* responded very differently. Like the cooperative species, it transferred more P to the root compartment with access to more C, showing it was able to assess and respond to the rate of C supply (Fig 2D). However, this species predominately stored the P resources in a host inaccessible form (long-chained polyphosphates (Takanishi *et al.*, 2009)(Fig. S6). This type of resource hoarding reduces P availability for competing fungi and P directly available for host uptake (Fig. S8), and illustrates key differences in fungal strategies, with *G. intraradices* being a ‘reciprocator’, and *G. aggregatum* a less-cooperative ‘hoarder’.

To track simultaneous resource exchange between partners, and hence test whether AM fungi are stimulated to provide more P in direct response to a greater host C supply, we used a two-compartment Petri plate design. Host roots were exposed to labeled U-^{14}C sucrose in either high or low concentrations, while labeled ^{32}P was added to the fungal compartment. We found that increasing C supply stimulated P transfer by the cooperative fungal species *G. intraradices*, but not the less-cooperative species *G. aggregatum* (Fig. 3A).

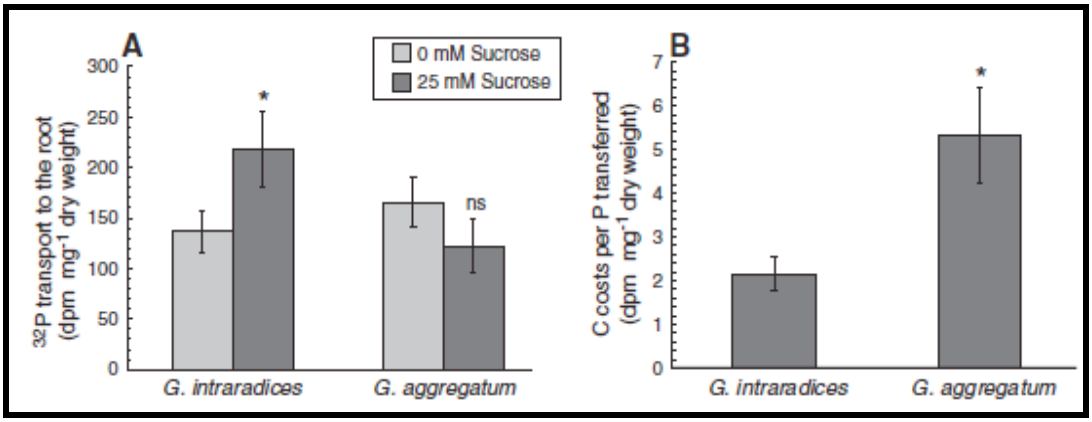

Figure 3. Simultaneous labeling of P and C exchange. (**A**) Higher C availability stimulated increased P transfer by the cooperative species, *G. intraradices* ($F_{3,22}$ = 3.07, *P*=0.0489), but not by the less cooperative species, *G. aggregatum*. (**B**) When supplied with 25 mM sucrose, the carbon costs per root P of *G. aggregatum* were more than 2 times higher than with *G. intraradices* ($F_{1,11}$= 8.27, *P* = 0.0151). Dpm=Disintegrations per minute. Asterisks indicate significant differences between treatment means (Student-Newman-Keuls test, $P \leq 0.05$). Bars represent means of 6-8 replicates ± 1 standard error.

As above, the cooperative species responded to C rewards with a reciprocal P increase, while the less-cooperative species utilized the extra C to increase P storage in the host inaccessible form (Fig. S7). Finally, we compared the ratio of C costs for P transferred in both species (Fig. 3B), confirming that colonization by the less-cooperative species resulted in significantly higher host costs. This result supports the findings of our SIP experiments (Fig. 1), and explains why the plant host consistently allocated more C to the cooperative species when given a choice.

Overall, our results suggest that stability of the AM mutualism arises in a significantly different way compared to other mutualisms. A general feature of many mutualisms is that one partner appears to be 'in control' (West & Herre, 1994), and has either domesticated the other partner (Poulsen & Boomsma, 2005), or enforces cooperation through punishment or sanction mechanisms (Leigh, 2010). In these cases, the potential for enforcement has only been found in one direction, with the controlling partner housing the other partner in compartments, which can be preferentially rewarded or punished, such as in legume root nodules (Kiers *et al.*, 2003), fig fruits (Jandér & Herre, 2010), and the flowers of yucca (Pellmyr & Huth, 1994) and *Glochidion* plants (Goto *et al.*, 2010). In contrast, the mycorrhizal mutualism involves both sides interacting with multiple partners so that neither partner can be 'enslaved'. Cooperation is only stable because both partners are able to preferentially reward the other. This provides a clear, non-human, example of how cooperation can be stabilized in a form analogous to a market economy, where there are competitive partners on both sides of the interaction, and higher quality services are remunerated in both directions (Noë & Hammerstein, 1995; Schwartz & Hoeksema, 1998; Bshary & Noë, 2003).

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2.5 SUPPORTING ON-LINE MATERIAL: RECIPROCAL REWARDS STABILIZE COOPERATION IN THE MYCORRHIZAL SYMBIOSIS

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2.5.1 MATERIALS AND METHODS

SELECTION OF FUNGAL STRAINS

We chose the three AM fungal species based on the following criteria: (**1**) all AM fungi belong to the same genus. By choosing closely related fungi, we were able to avoid problems associated with contrasting life history traits not necessarily associated with mutualistic benefit (Hart & Reader, 2005; Maherali & Klironomos, 2007; Powell *et al.*, 2009). (**2**) The fungi differentially affected growth of their host plant and this difference was evident within 10 weeks of growth (Fig. S3). Although fungal benefits could potentially change (increase or decrease) over the host's ontogeny (Fitter, 1991; Husband *et al.*, 2002; Smith *et al.*, 2009), we were interested in documenting early-stage fungal and host allocation patterns, in which there were fewer constraints on fungal and plant growth. At this stage, clear allocation patterns are predicted because resources acquisition demand is at its highest (Hoeksema & Kummel, 2003). (**3**) The benefits conferred to hosts were consistent across different plant species (Fig. S3). This allowed us to reject the hypothesis that the observed differences in mutualistic benefit were attributed to local co-evolutionary dynamics between host and fungal symbiont (Antunes *et al.*, 2011). (**4**) The

selected AM fungi differed in growth benefit but were not 'parasites' (see (Smith *et al.*, 2003; Ryan *et al.*, 2005; Smith *et al.*, 2009; Smith & Smith, 2011) for useful discussion). In our case the biomass of the plants inoculated with the less-cooperative AM fungal species was either equal, or greater than the growth of the non-mycorrhizal control plants (Fig. S3). This allowed us to examine whether hosts could detect and respond to variation in fungal cooperation (Jansa *et al.*, 2005; Hodge *et al.*, 2010), rather than testing for host response to a negative growth impact (e.g. a non-cooperative species (Ryan *et al.*, 2005)). (**5**) We utilized species with different structural patterns. At 10 weeks, *G. custos* allocated significantly more to spore production, and *G. aggregatum* allocated significantly more to vesicles compared to the other two AM fungal species (Fig. S4). The use of these two less-cooperative species allowed us to test for host response when the choice was binary (*G. intraradices* versus *G. aggregatum*), and also test for host response in AM communities with three species, which included two less-cooperative species, *G. aggregatum* and *G. custos* differing in their carbon storage strategies. In these less cooperative fungi, high spore and vesicle formation are potential indicators of fungal resource hoarding. Ratios of these fungal storage units to arbuscules (nutrient transfer structures) are often used as an estimate of symbiotic effectiveness (Johnson *et al.*, 1992; Johnson, 1993; Johnson *et al.*, 2003). (**6**) Importantly, we do not categorize our less-cooperative species as unequivocal 'cheats' (Smith *et al.*, 2003; Douglas, 2010; Smith & Smith, 2011). AM fungi can confer diverse benefits to the host plant (protection against pathogens, drought, or heavy metal uptake) not measured here (Sikes *et al.*, 2010). It is well known that biotic and abiotic changes can alter the relative benefits of AM fungi (Hoeksema *et al.*, 2010). No experimental design can explore all the diverse conditions

under which the relationship with particular fungi is potentially beneficial (Helgason & Fitter, 2009). (**7**) To increase the ecological context of our experimental design, all fungal species were isolated from temperate ecosystems between 37- 43° degrees, and from areas in which Medicago sp. hosts are found. While these species are globally cosmopolitan, it is well known that fungal isolates –within a species - can differ greatly in the benefits they confer to their hosts (Koch *et al.*, 2006). While it would be interesting to conduct future experiments that utilize plant and fungal material collected from a single ecosystem, we note that there are difficulties in isolating fungal strains from one location that meet all our criteria for selection (see **criteria 1-6** above).

FUNGAL CULTURES

For all experiments, we produced inoculum of *Glomus intraradices* (Schenck & Smith; isolate 09 collected from Southwest Spain by Mycovitro S.L. Biotechnología ecológica, Granada, Spain), *G. custos* (Cano & Dalpé; isolate 010 collected from Southwest Spain by Mycovitro S.L.) and *G. aggregatum* (Schenck & Smith; isolate 0165 collected from the Long Term Mycorrhizal Research Site, University of Guelph, Canada) by growing the fungus in association with Ri T-DNA transformed carrot (*Daucus* carota clone DCI) roots in Petri dishes filled with mineral medium (St-Arnaud *et al.*, 1996) and with sucrose as the only carbon source. We cultured roots for approximately 8 weeks (until the plates were fully colonized) and fungal spores were isolated from the growth medium by solubilising the medium with 10 mM citrate buffer (pH 6.0).

DESIGN OF SPECIES-SPECIFIC QUANTITATIVE REAL-TIME PCR (QPCR) MARKERS (PRIMERS AND HYDROLYSIS PROBES)

To quantify the abundance of each AM fungal species in the stable isotope probing (SIP) experiments, we designed markers targeting species-specific motifs in the mitochondrial large ribosomal subunit RNA genes of *G. intraradices*, *G. aggregatum* and *G. custos*.

DNA PREPARATION AND AMPLIFICATION

We extracted fungal DNA from both spores and colonized roots produced monoxenically, as described below. We used DNeasy Plant Mini kit (Qiagen, Hombrechtikon, Switzerland) and followed the recommendations of the manufacturer with slight modifications. For spores only, the final volume of the DNA preparations was 20 µl (instead of recommended 100 µl) to maximize DNA concentration before PCR. DNA was subjected to PCR amplification of the mitochondrial large ribosomal subunit (mtLSU) RNA gene with following primer pair combinations, RNL11-RNL17, RNL1-RNL14, or RNL1-RNL15 (according to (Börstler *et al.*, 2008)). The PCR was carried out using Taq PCR Core kit with CoralLoad reaction buffer (Qiagen), using a 25 µl PCR reaction volume, 1 µM of each primer, and 38 cycles (denaturation at 95°C for 10 s, annealing at 50°C for 90 s and amplification at 72°C for 90 s). Amplified DNA fragments were cloned into a blue-script vector (pGEM-T Easy vector system; Promega, Dübendorf, Switzerland) and sequenced by Microsynth AG (Balgach, Switzerland). The sequences were individually edited and the clones re-sequenced if the quality of the reads proved to be insufficient. The identity of the sequences was revealed by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to exclude potential contaminant sequences (e.g.,

bacteria, unspecific amplifications of other genome regions).

PROBE DESIGN

The sequences of our three AM fungal species were aligned with other available mtLSU sequences from e.g. *G. intraradices*, *G. proliferum* and *G. clarum* in order to construct our hydrolysis probes. For each fungal species at least two species-discriminating primers with associated hydrolysis probes were designed using the AlleleID software (version 6, Premier Biosoft International, Palo Alto, California, USA). Care was taken to target mtLSU regions coding for the ribosomal RNA to avoid putative introns described recently (Thiéry *et al.*, 2010). We confirmed the specificity of the primers and fluorescent probes with a BLAST search and the oligonucleotides (primers and dually labeled hydrolysis probes, labeled with fluorescein at the 5`-end and BHQ-1 quencher at the 3`-end) were then synthesized by Microsynth AG (Balgach, Switzerland). Primers and probes were purified by preparative HPLC or preparative polyacrylamide gel electrophoresis, respectively, before lyophilization. Both primers and probes were diluted with PCR-grade water to achieve 25 µM concentrations, aliquoted (20 µl each) and frozen at -20°C.

PRIMER SELECTION, OPTIMIZATION OF CYCLING CONDITIONS, CROSS-REACTIVITY TESTING (DNA AND cDNA)

To ensure species-level specificity, we performed several optimization steps. First, we tested the markers for specificity under low stringency cycling conditions (denaturation at 95°C for 10 s, annealing at 52°C for 30 s, and amplification at 72°C for 5 s). In this assay,

we used DNA extracts from *M. truncatula* roots colonized by the different AM fungi (3 replicates for each species) as templates. From this initial test, primer pairs and probes showing greatest specificity towards their target species (either no cross-amplification with other species or the greatest difference in Cq value between target and non-target species) were selected for further optimization (see Table S1). Stringency of cycling conditions was then increased stepwise for each of the markers to avoid amplification of non-target samples (see Table S1 for details of the optimized cycling conditions and Table S2 for the results of the cross4 amplification assay). Finally, to confirm that the markers only amplified the target fungal species, and that they avoided plant genes and were suitable at the RNA level, we performed another cross-amplification assay using cDNA generated from RNA extracts of non-mycorrhizal or mycorrhizal roots of *M. truncatula* colonized by the different fungal species (Table S2). Again, all three markers were confirmed to be species-specific at both, DNA and RNA level.

qPCR CALIBRATION AND DETECTION LIMITS

We generated plasmids carrying fragments of the mtLSU of the respective fungal species with 100% sequence match to the region amplified in order to: (1) to calibrate the qPCR detection cycle (Cq) with the gene copy concentrations and (2) to assess the detection limits of the qPCR markers. Cq is typically negatively and linearly correlated to the log-transformed template concentration (linear response region), until the detection limit of the assay is reached and the Cq becomes independent of the further dilution (background region) (Fig. S9), or there is no response at all. We used the linear response region of each calibration assay to derive equations that allowed the conversion of Cq

values to mtLSU gene copies per unit volume of the template (Fig. S10). The detection limits were calculated from the background region of the qPCR response curve as follows:

$$DL = AV_{cq(back)} - 3 \times SD\ (AV_{cq(back)})$$

where DL represents the detection limit of the assay (Cq value), $AV_{cq(back)}$ the mean of the Cq values in the background region and SD ($AV_{cq(back)}$) the standard deviation of this mean. The detection limits of the three assays and the corresponding threshold concentrations of mtLSU are given below (Table S3). These assays were then used to determine the mtLSU gene copy concentration in DNA and cDNA samples, fractionated or not by ultracentrifugation, and taking into account any dilutions of the template during sample processing.

PLASMID PREPARATION

Between two and four individual plasmid preparations per fungal species were used for the calibration of the qPCR markers. The plasmids were isolated from overnight cultures of transformed *E. coli* JM109 cells (Promega, Madison, WI, USA), grown on LB medium supplemented with 100 µg ml-1 Ampicillin, using the Miniprep procedure (Sambrook *et al.*, 1989). The plasmids were linearised using the EcoRI+ digestion (Fermentas, Le Mont-sur-Lausanne, Switzerland) at 37°C for 2 h and then at 65°C for 20 min. The concentration of the DNA was then measured by the PicoGreen fluorescence assay (P7589, Invitrogen, San Diego, CA, USA), using Roche LightCycler 2.0 at 45°C and measuring the emission at 530 nm. The concentration of plasmid copies per unit of sample volume was calculated according to (Jansa *et al.*, 2008) under consideration of the

DNA concentration in each sample, the length of the insert (176 bp for *G. intraradices*, 661 bp for *G. aggregatum*, and 438 bp for *G. custos*) and vector (3015 bp), and an estimated molecular weight per nucleotide double-stranded DNA of 660 Da. Plasmid preparations were serially diluted (5-fold and 10-fold) to achieve a range of plasmid concentrations from a few billions to (theoretically) less than 1 per microliter.

STABLE ISOTOPE PROBING

PLANT CULTURE

Seeds of *Medicago truncatula* (variety Jemalong A17, courtesy of Bettina Hause, Leibniz Institute of Plant Biochemistry, Halle, Germany) were pre-treated with concentrated H_2SO_4 and exposed to a cold treatment (4°C in the fridge) for 3 days. The seedlings were transferred to a sterilized peat-based growth medium for 5 days and then planted in 1 L pots filled with sterilized nutrient-poor dune sand with the following characteristics: pH 7.2; 0.2% organic matter; 0.3 mg kg^{-1} P(CaCl2-extracted) and 190 mg kg^{-1} total N.

For the two-species experiment, the seedlings were inoculated at planting with 1500 spores per plant and 1.0 g of in vitro root material of either *G. intraradices* or *G. aggregatum* (singles) or both species together (mixed 50:50) with inoculum concentrations reduced by half. For the three-species experiment, *G. custos* was included in the mixed treatment and the inoculum concentrations of the three AM species were reduced to one-third each. We assumed that in this mixed treatment, the nutrient-acquiring strategies of our AM species were fixed, meaning that strategies did not undergo fundamental change (switch from less-cooperative to cooperative or vice versa)

simply because other symbionts were present (Kiers & Denison, 2008).

Non-mycorrhizal control plants were inoculated with autoclaved inoculum. Plants were grown in a greenhouse with a 13 h light cycle. When the outside daylight was below 120 J cm^{-2} h^{-1}, supplemental lights of 15,000 lux, were turned on. The temperature was kept between 22 and 25°C. Soil humidity was maintained at 70% water holding capacity and nutrients (8 ml per pot of Hoagland solution (Arnon & Hoagland, 1940) containing only 50% of original P concentration) were added every two weeks. The plants were grown for a total of 10 weeks before $^{13}CO_2$ labeling.

$^{13}CO_2$ LABELING AND HARVEST

Plants were labeled with $^{13}CO_2$ at the Experimental Soil Plant Atmosphere System (ESPAS, Isolife, Netherlands) (Gorissen *et al.*, 1996), with a day/night rhythm of 16/8 h and at 21°C and 17°C, respectively, an irradiation of 700 µmol m^{-2} s^{-1} at plant height, and 80% relative humidity. The plants were acclimated to the chamber for 48 h before labeling. The mean CO2 level in the chamber was maintained at 401±19 µl l^{-1} by injection of $^{12}CO_2$ from a pressurized cylinder. During the night period prior to labeling, $^{12}CO_2$ was removed by a CO_2-scrubber in accordance with the $^{12}CO_2$-respiration of *M. truncatula.* One hour before the start of the day period, $^{13}CO_2$ was injected from a pressurized cylinder (99 atom % ^{13}C, 1 atom % ^{12}C; Isotec, Inc. Miamisburg, OH, USA). For 6 h, a total CO_2-level ($^{12}CO_2$ + $^{13}CO_2$) of 396±20 µl l^{-1} CO_2 was maintained. The ^{13}C-enrichment of the atmosphere was 92% at the start of the 6-h labeling period. This value gradually decreased due to the $^{12}CO_2$ respiration by the plant and resulted in a mean ^{13}C-enrichment of 86.5±3.0 % over the time course of labeling.

In both the two-species and three-species experiment, the labeling chamber was opened and flushed with fresh air after 6 h to remove the labeled $^{13}CO_2$. After the flushing period, the labeling chamber was closed and the $^{12}CO_2$ level was maintained at 405±29 μl l^{-1}. To follow the incorporation of ^{13}C label over time in the two-species experiment, replicate plants were harvested at the 6 h-flushing period and again 6 h later at the 12 h time point. In the three species experiment an extra harvest time was added, so plants were harvested at 6 h, 12 h and 24 h. In both experiments, all replicates of the single inoculated control treatments were harvested at the 6 h time point. At each harvest, the aboveground plant parts were removed, oven dried at 70°C for 72 h, and weighed. The root systems were gently washed, weighed, homogenized and five root aliquots were placed in Eppendorf tubes and frozen with liquid N_2. A small subsample of roots was removed, processed in 10% KOH, and stained with trypan blue to quantify the mycorrhizal colonization and fungal structures in the root (McGonigle *et al.*, 1990). Sand was collected and spores were counted using conventional decanting and wet sieving methods (Gerdemann & Nicolson, 1963).

RNA EXTRACTION, ULTRACENTRIFUGATION, AND CDNA SYNTHESIS BY REVERSE TRANSCRIPTION

RNA was extracted from roots using the RNeasy Plant Mini Kit (Qiagen, Hombrechtikon, Switzerland), tested for quality and RNA concentration using a Nanodrop1000™ and stored at -80°C. For centrifugation, 500 ng of RNA was transferred in 2 ml ultracentrifuge tubes (Sysmex, Kobe, Japan) pre-filled with 1.99 ml of 1.8 g ml^{-1} CsTFA solution. The samples were then placed into a Sorvall discovery m120 SE micro ultracentrifuge (Thermo Fisher Scientific, Waltham, MA, USA) with a S120VT fixed

angle titanium vertical rotor for 48 h at 20°C at a speed of 64000 rpm, resulting in a gravity of 142,417 g at the maximum radius and 91,1128 g at the minimum radius. Between 17 to 20 fractions of 100 µl each were collected from every 2 ml vial. To remove these fractions, the tubes were punctured at the bottom and top using a needle. The upper needle was connected to a syringe pump (Harvard Apparatus, Kent, UK) that allowed a continuous flow rate (220 µL min^{-1}) of RNAse free water. This initiated a continuous flow of fractions from the lower needle. An extra vial was included in each ultracentrifugation batch for gravimetric estimation of density of each gradient fraction in each ultracentrifugation run (60). The RNA in each fraction was precipitated, dried and resuspended in 15 µl of ultrapure water. Five µl were then used for reverse transcription (RT), using a final volume of 25 µl and the following reaction components: 5 µl 5xRT buffer, 1.5 µl of 10 mM dNTPs, 0.5 µl random hexamers, 1 µl of 200 u/µl, MMLV reverse transcriptase (Promega Corp., WI, USA) and 12 µl water.

REAL TIME QUANTITATIVE PCR (QPCR) ANALYSIS

All qPCR assays were carried out in 9 µl reactions, using the LightCycler 2.0 instrument, LightCycler TaqMan chemistry (LightCycler TaqMan Master) and 20 µl-Lightcycler glass capillaries. The final concentrations of the primers and the hydrolysis probe were 0.5 µM and 0.11 µM, respectively (for sequences see Table S1). Each reaction included 2.25 µl of the DNA template (i.e. sample).

QUANTIFICATION OF RNA ABUNDANCE OF THE DIFFERENT FUNGAL SPECIES

To quantify the enrichment of fungal RNA with host derived ^{13}C in the different fractions, we used qPCR targeting species-specific sequence motifs in the mtLSU, as described above. All reactions were carried out separately, not multiplexed, under stringent cycling reaction (Table S1). Briefly, 2.25 µl of the RT reaction (see above) was used as a template for qPCR, and the total qPCR reaction volume was 9 µl. Gene copy concentrations were calculated per µl template using the quantification cycle (Cq) from each assay and the respective calibration curves (Fig. S10). The results of mtLSU quantification of each AM fungal species in the different fractions were subjected to nonlinear regression, using the Gaussian, 3-parameter function option in SigmaPlot for Windows version 11.0. This function is described by the following formula:

$$y = a \times e^{(-0.5 \times \left(\frac{x - x_0}{b}\right)^2)}$$

where a and b are constants, x_0 is the x value of function peak, and e is the base of natural logarithm (approximately 2.718). Only the samples with R^2 of all relevant regressions higher than 0.64 (i.e., $R \geq 0.8$) were used for subsequent statistical analyses. This data selection was necessary in order to exclude samples that suffered high RNA degradation during ultracentrifugation and subsequent steps, and/or poorly fractionated samples, where the gradients were obviously disturbed during fraction collection. This quality check resulted in the removal of 1 out of 12 samples in the two-species experiment, and 6

out of 17 samples in the experiment with three AM fungal species.

ANALYSIS OF PEAK FRONT

Variation in host C allocation patterns were calculated based on differences in 'peak front' among AM species. Peak front is the position (i.e. density in mg ml^{-1}) of the heaviest RNA fraction of each of the AM fungal species. Each fungal species shows a unique peak front position that can be compared against the others. Peak front is defined mathematically as the foremost fraction of the Gaussian regression curves cutting through the detection limit of the qPCR assay. Peak fronts further to the left (see Fig. S2 for example) mean higher ^{13}C enrichment, indicative of preferential C allocation to that fungal species. To determine peak front differences among the AM fungal species within each individual plant sample, we first measured abundance of each AM fungal species (copies of mtLSU) in each RNA density fraction by using qPCR with species-specific markers (Table S1). Then, Gaussian regressions across the different fractions were constructed for each AM fungal species. Peak fronts for the different AM fungal species were compared only when meeting requirements listed above, thus removing technically imperfect samples from statistical comparison.

To determine if there were significant differences in ^{13}C enrichment of our AM fungal species, we ran pair-wise comparisons of peak front position for all pairs of AM fungal species. We calculated differences in peak front positions based on a non-parametric sign test, using Statgraphics Plus software (version 3.1 for Windows). *P*-values (Fig. 1) refer to differences of the sample median from zero, with values above zero indicating preferential allocation to that particular fungal species.

To further confirm our preferential allocation findings, we ran additional analyses using a parametric generalized linear model (GLM) approach. For each replicate and each fungal species combination, differences in peak front positions between AM fungal species were calculated, as described above. A GLM was produced independently for both the two-species and three-species experiments to test the variables of differential 13C enrichment and harvest time. The Akaike criteria (AIC) was used to select the optimal GLM, which in our case was in the gamma family. A 'saturated model' reproduced the observed data. The relative importance of a given interaction term or a co-variable was estimated after removal of this term from the saturated model. Deviance analyses using Fisher tests were performed. Using this approach, we confirmed our finding that the RNA of the cooperative species (*G. intraradices*) was significantly more enriched than that of the two less-cooperative species (*G. aggregatum* and *G. custos*). We found significantly higher ^{13}C enrichment in both the two-species experiment (*G. intraradices* vs. *G. aggregatum*, $P = 0.019$) and in the three species experiment (*G. intraradices* vs. *G. aggregatum*, $P= 0.030$) and (*G. intraradices* vs. *G. custos*, $P = 0.016$). There was no significant difference in RNA enrichment of the two less-cooperative species (*G. aggregatum* vs. *G. custos*, $P > 0.05$). The GLM deviance analyses showed no significant effect of time on allocation patterns for both the two-species ($P=$ 0.4267) and three-species ($P= 0.5571$) experiments. All GLM analyses were carried out using the program R (http://www.r-project.org/).

ANALYSES OF NON-FRACTIONATED RNA SAMPLES

The non-fractionated RNA samples were reverse transcribed and the cDNA was used as template for qPCR quantification of mtLSU copies as described above. The results were converted to mtLSU RNA copies per 500 ng RNA. These results were used to compare the abundance of the different fungal species in the roots after inoculation with single or mixed AM fungal species (Fig. S5).

MANIPULATION EXPERIMENTS WITH IN-VITRO ROOT ORGAN CULTURES

For all resource manipulation studies, we used Ri-T-DNA-transformed carrot roots (*D. carota* L., clone DCI), that were colonized with the cooperative AM fungus *G. intraradices* or the less-cooperative AM fungus *G. aggregatum*. These two fungal species were used for the resource manipulation experiments because they differed greatly in cost to benefit ratios for P to C exchange (~2.5 higher C costs in *G. aggregatum*, Fig. 3A), and represented the maximum and minimum of the host benefit continuum (Frank, 1996; Egger & Hibbett, 2004; Jones & Smith, 2004).

While root organ cultures (ROCs) have been criticized for their artificial nature (Fortin *et al.*, 2002), it has been repeatedly demonstrated that ROCs possess similar nutrient and resource transfer and metabolic characteristics as whole plant systems (Pfeffer *et al.*, 2004). ROCs have been pivotal in producing a large body of literature that has shaped our understanding of nutrient transport and C exchange in the AM symbiosis (Olsson *et al.*, 2002; Bago *et al.*, 2003; Bücking & Shachar‐Hill, 2005; Govindarajulu *et al.*, 2005; Jin *et al.*, 2005; Olsson *et al.*, 2005; Olsson & Johnson, 2005; Tian *et al.*, 2010; Hammer *et*

al., 2011). ROC model systems offer a number of important advantages for our study, including (**1**) the separation into fungal and root compartments (which prevented the diffusion and exchange of substrates between the compartments) and thereby precise control over quantities of resources supplied to fungus and host, (**2**) high visibility of the system, allowing us to select comparable plates for each experiment regarding e.g. the colonization of the fungal compartment, and (**3**) precision with which the ERM could be collected. In addition, ROCs provide the ideal model system for comparing particular traits (e.g. N or P transfer) across AM species, while standardizing for confounding environmental factors. This allowed us to compare baseline functioning and then manipulate resources to test for host and fungal responses to nutrient availability. Such small-scale manipulations are not yet possible in a soil based system. In the future, *in-vitro* whole-plant systems could be a useful test system for biological market experiments with AM fungi (Gyuricza *et al.*, 2010). However, the challenge of working with *in-vitro* whole plants is the loss of precision in controlling the carbohydrate allocation from the host to the fungus. Although manually shading plants can be utilized as a potential treatment to reduce host C, the effects are difficult to control and to quantify, and secondary effects of the reduced photosynthetic rate on plant physiology cannot be excluded.

IN VITRO ROOT ORGAN CULTURES

We grew mycorrhizal systems in Petri dishes with two or three compartments (depending on the experimental design) at 25°C. The mycorrhizal roots were confined to one or two root compartments (St-Arnaud *et al.*, 1996) filled with solidified mineral medium (Chabot *et al.*, 1992) containing 10 g l^{-1} sucrose. AM fungi are obligate

biotrophs that cannot use this C source directly but rely on carbon that is supplied by the host. After approximately eight weeks of growth, the colonized root compartments were transferred into new Petri dishes and the extraradical mycelium (ERM) of the fungus was allowed to cross over the divider into one or two fungal compartments (depending on the experimental design, see below). These fungal compartments were filled with solidified mineral medium without sucrose and phosphate addition (KH_2PO_4 was replaced with an equimolar concentration of KCl). After approximately 3 weeks, the fungal compartments were sufficiently colonized by ERM and the plates could be used for the experiments.

EXPERIMENTAL DESIGN OF THE ROC EXPERIMENTS

PREFERENTIAL CARBON TRANSPORT FROM COLONIZED ROOTS TO FUNGAL ERM COMPARTMENTS DIFFERING IN P SUPPLY.

Here, we asked the question: Will hosts transfer significantly more C to the fungal hyphae with access to more P (Fig. 2A,B)? We tested this question when hosts were colonized either by the cooperative species *G. intraradices* or the less-cooperative species, *G. aggregatum*. We used a three compartment Petri dish design with one mycorrhizal root compartment and two fungal compartments differing in P supply. Labeled sucrose (22.2 mM sucrose containing [U-14C]sucrose, 1:500000, v/v) with a specific activity of 498 mCi mM^{-1} (Sigma-Aldrich, St. Louis, USA) was supplied to the root compartment. Simultaneously, water (0 µM P) was added to one fungal compartment and 35 µM P or 700 µM P (as KH_2PO_4) to the other fungal compartment. After 4 days, 6 replicates per treatment were harvested and processed for liquid scintillation counting (see below).

PREFERENTIAL P TRANSPORT FROM THE ERM TO ROOT COMPARTMENTS DIFFERING IN C SUPPLY.

Here, we asked the question: will significantly more P be transferred to the root compartment with access to more C (Fig. 2C,D)? Again, we tested this with the cooperative species *G. intraradices* and the less-cooperative species, *G. aggregatum*. We used a reciprocal design of the three-compartment Petri dish system described above, now with two root compartments and one fungal compartment. This allowed us to track the transport of P from the fungal ERM to colonized roots that differed in their carbon supply. Fungal hyphae from both root compartments were allowed to cross over into one root-free compartment. When approximately the same number of hyphae had crossed over from each root compartment into the fungal compartment, 6.4 μCi [33P]orthophosphate (Perkin Elmer, Waltham, USA) and 35 μM non-labelled KH_2PO_4 were added to the fungal compartment. The carbon supply in the root compartments was varied at the same time by adding 0.5 ml of water to one root compartment (0 mM control) and 0.5 ml of a sucrose solution to reach 5 mM or 25 mM in the other root compartment. After 4 days, 6 to 10 replicates per treatment were harvested and prepared for further analysis (see below).

SIMULTANEOUS MEASUREMENTS OF SYMBIOTIC EFFECTIVENESS AND CONDITIONAL RESPONSE.

Here we asked two questions: (**1**) Does increasing host C supply lead to an increase in P transfer by both the cooperative and less-cooperative fungal symbionts (Fig. 3A) and (**2**) does the baseline cost to benefit ratios (here in terms of carbon costs for P supplied to the root) differ between the two fungal species (Fig. 3B)? To achieve both these aims, we used a two compartment Petri dish system with one root and one fungal compartment to which simultaneously ^{14}C–sucrose and ^{32}P-orthophosphate were added. Three weeks after the ERM started to cross over the divider, we added [U-^{14}C] sucrose with a specific activity of 498 mCi mM-1 (Sigma-Aldrich, St. Louis, USA) to the root compartment and [^{32}P] orthophosphate (Sigma-Aldrich, St. Louis, USA) to the fungal compartment. To test for differences in P transport in response to increasing C supply and determine the cost to benefit ratio of each fungal species, one set of plates was only supplied with ^{14}C labeled sucrose and ^{14}C labeled sucrose diluted with non-labeled sucrose for a final sucrose concentration of 25 mM sucrose (0.448 μM as ^{14}C labeled sucrose) was added to the other set. After 4 days, 8 replicates per treatment were harvested and prepared for further analysis (see below).

LIQUID SCINTILLATION COUNTING

For all experiments, we harvested the mycorrhizal roots and the fungal ERM after 4 days of labeling. The ERM was isolated from the medium in the fungal compartment after several wash and centrifugation steps in Na citrate buffer (10 mM, pH 6.0). An aliquot of the medium was taken to determine the radioisotope residues in the medium

and to confirm that there were no cross-contaminations between compartments in the plates. The root and ERM samples were dried in an oven at 70°C, weighed and digested with a tissue solubilizer (TS-2, rpi corp., Mount Prospect, USA). The radioactivity was determined by liquid scintillation counting (Wallac, Perkin Elmer, Waltham, USA) using standard full channel programs in single isotope experiments or by channel settings that allowed the differentiation of ^{14}C and ^{32}P according to the emission energy in dual isotope experiments. The ^{14}C measurements in the dual isotope experiments were additionally confirmed by measuring the samples for a second time 4 months later (i.e., after 8 half-lives of ^{32}P passed), when ^{32}P was sufficiently depleted. The accuracy of all measurements was corrected by use of an internal standard.

EXTRACTION OF VARIOUS PHOSPHATE POOLS AND ANALYSIS OF PHOSPHATE POOL DISTRIBUTION

To examine the phosphate pool distribution in mycorrhizal roots which were supplied with varying concentrations of sucrose (Fig. 2C,D, Fig. S6, S7), we extracted phosphate pools according to the method described previously (Aitchison & Butt, 1973). The following phosphate pools were extracted and could be distinguished: (**1**) inorganic orthophosphate and acid soluble or short-chained polyphosphates (chain length of less than 20 Pi residues) after extraction with 10 % TCA (w/v) at 4°C (two times); (**2**) phospholipids after extraction with first 100 % ethanol and then ethanol:ether (3:1, v/v), (**3**) acid insoluble or long-chained polyphosphates (chain length of more than 20 Pi residues) after extraction with 1 M KOH at room temperature (two times), and (**4**) DNA-, RNA- and protein-phosphates (residue). Acid soluble polyP (short chain length) and acid insoluble polyP (long chain length) within the supernatants were precipitated two times

by a saturated $BaCl_2$ solution over night at 4°C. We used polyP pools to measure P transport, because both polyphosphate pools are of fungal origin (plants are not able to produce polyP) and better represent P transport from the ERM to the IRM. The ^{33}P content in all fractions was determined by liquid scintillation counting.

2.5.2 STATISTICAL ANALYSIS

Data from the ROC experiments were analyzed using Unistat Software, P-STAT Inc. (Hopewell, NJ, USA). For all experiments, the data were subjected to a variance analysis (ANOVA), with 'resource-level' as the treatment factor. Disintegrations per minute (dpm) values after scintillation counting were log transformed before the analysis. Following significant ANOVA, treatment means were compared using the Student-Newman-Keuls test ($P \leq 0$ .05).

2.5.3 TEXT

We conclude by raising three important points: (**1**) our work does not preclude the possibility that partners employ other mechanisms to control the growth/success of each other. Various mechanisms have been proposed to explain, for example, how mycorrhization may be mediated by host plants (Pearson *et al.*, 1993; Johnson *et al.*, 1997; Blee & Anderson, 1998; Vierheilig, 2004; Schaarschmidt *et al.*, 2007; Bonfante & Genre, 2010). One possible mechanism is the digestion of fungal arbuscules by plant hosts (Kobae & Hata, 2010). Although alternative explanations for premature arbuscular death cannot yet be ruled out (Smith & Smith, 2011), empirical work has demonstrated

that the lifespan of an arbuscule may be related to its ability to deliver P (Javot *et al.*, 2007) or to the P status of the host (Breuillin *et al.*, 2010). Molecules such as lysophosphatidylcholine (LPc) have been suggested to be involved in P sensing and gene regulation in plants, potentially allowing hosts to evaluate the amount of P delivered via the mycorrhizal pathway (Bucher *et al.*, 2009). As more genome information becomes available, the molecular mechanisms governing the resource-sensing and control processes of both partners will be elucidated (Bucher, 2007). (2) Here, we demonstrated the importance of P as a resource for determining trade dynamics (e.g. (Pearson & Jakobsen, 1993)), but allocation based on other fungal commodities such as N, may likewise be important (Tanaka & Yano, 2005; Atul-Nayyar *et al.*, 2009; Hodge & Fitter, 2010). Research is now needed to determine how resource stoichiometry (e.g. the relative availability of carbon, nitrogen and phosphorus) affects trade among partners. (**3**) Although our work demonstrated that trade is favored with partners offering the best rate of exchange, this finding does not imply equal control in the mutualism. It is well-known that at high P levels: (i) the mycorrhizal nutrient uptake pathway can be repressed (Nagy *et al.*, 2009), (ii) root exudate activity to stimulate presymbiotic growth of AM fungi is reduced (Gadkar *et al.*, 2003), and (iii) the host may degrade the arbuscules of the fungus (Breuillin *et al.*, 2010) . In contrast, AM fungi are obligate biotrophs, meaning they will always rely on hosts for C. The implication is that, although fungi may choose to transfer P to the plant offering the highest C benefit, they will always need a host plant to complete their life cycle.

2.5.4 FIGURES

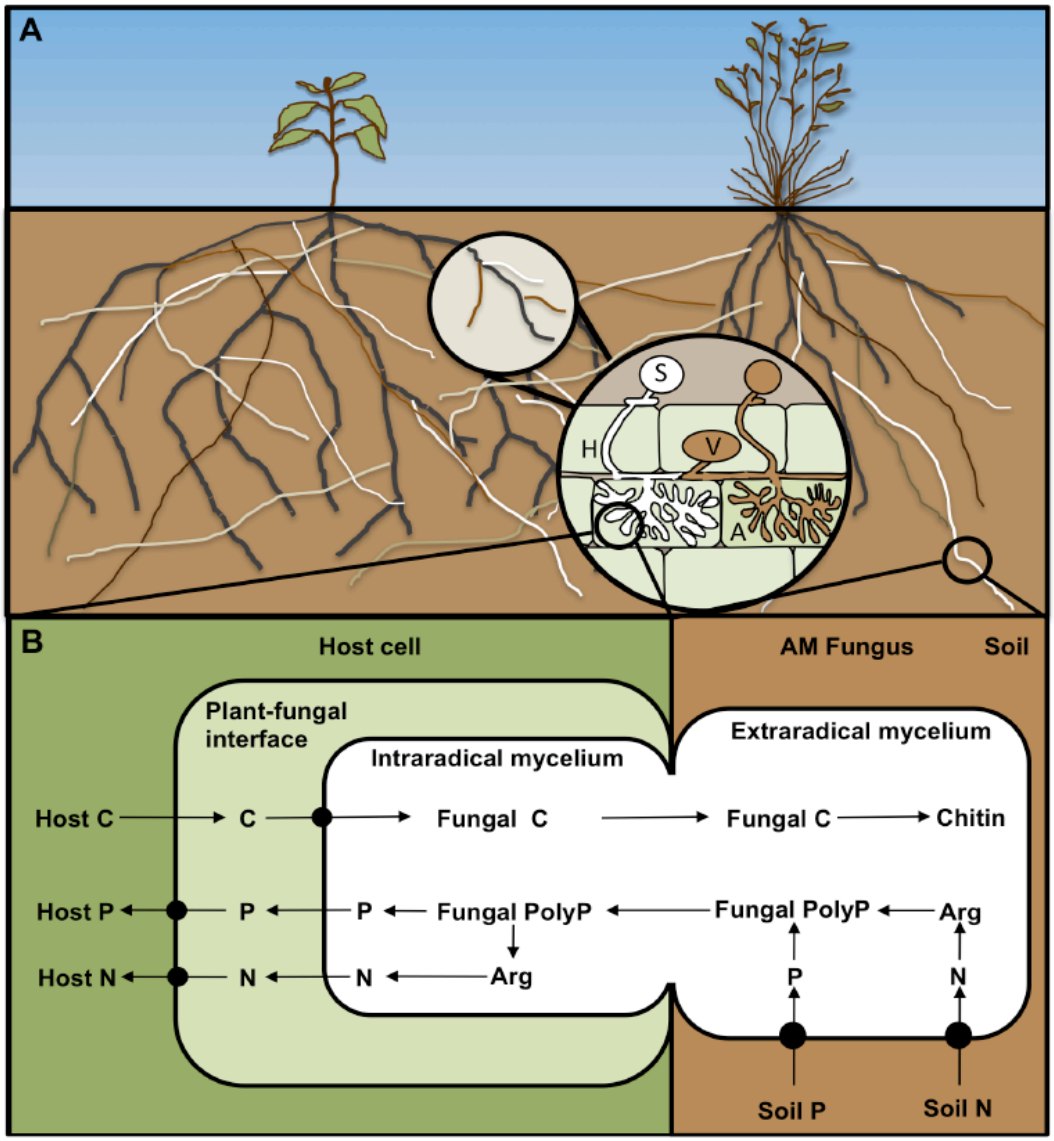

Fig. S1: Schematic drawing of the arbuscular mycorrhizal (AM) mutualism and resource exchange processes. (**A**) Land plants interact with diverse AM fungal communities (different species/strains represented by different colors) and AM fungi interact with multiple host plants. The mutualism is characterized by an exchange of mineral nutrients (e.g. N and P) from the fungus for C from the host plant. The transfer of nutrients occurs primarily across specialized structures called arbuscules (a). Fungal carbon is allocated to hyphae (h), vesicles (v) and/or spores (s). (**B**) Nutrient exchange between plant and fungal partner. Host C is transferred across the plant-fungal interface, taken up by the fungus and translocated to the extraradical mycelium (ERM). P is taken up from the soil as inorganic P (Pi) and converted into polyphosphates (PolyP). PolyP plays a key role in transferring nutrients to the Intraradical mycelium. Nitrogen, as NH4 and NO3, is likewise absorbed from the soil by AM fungi, and assimilated mainly into arginine (Arg). PolyP are negatively charged polyanions that can also bind the basic amino acid Arg. In the intraradical mycelium, PolyPs are remobilized and release inorganic phosphate (P_i) and Arg. Arg is further broken down to inorganic N (specifically NH4 +), and then transferred across the plant-fungal interface.

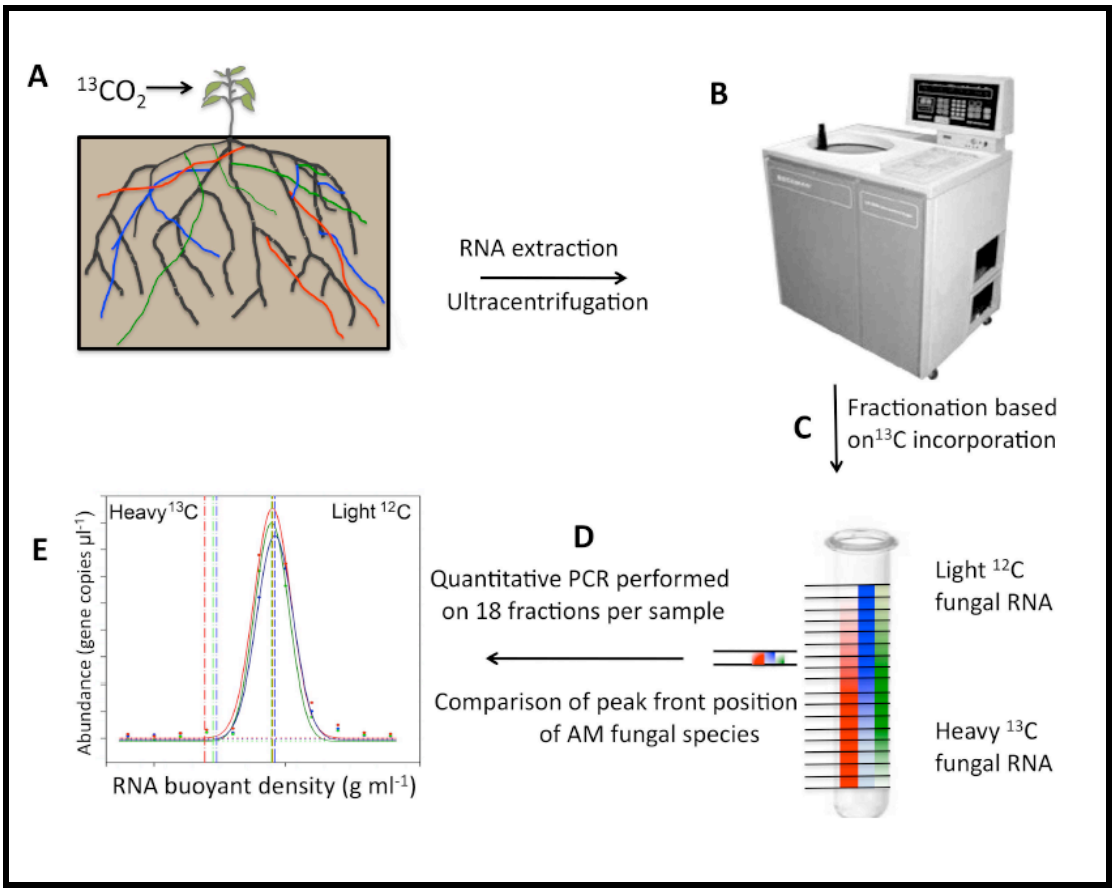

Fig. S2: The detection of plant-derived C fluxes into microbial nucleic acids by stable isotope probing (SIP). (**A**) Plants were inoculated with three fungal species (red, blue, green). The plants were labeled with $^{13}CO_2$ that was then incorporated into the RNA of the AM fungal community. (**B**) After extraction, the fungal RNA was ultracentrifuged in a cesium trifluoroacetate gradient. (**C**) The ultracentrifugation fractionated the RNA in layers based on the relative amount of ^{13}C-labeled carbohydrates incorporated by each fungal species. (**D**) Each centrifuge tube was punctured at the bottom and fractions (~18 per replicate) of 100 µL were taken using a long needle. The abundance of each AM fungal species was then quantified in every fraction using qPCR with species-specific markers targeting the mitochondrial large ribosomal subunit. (**E**) Results from the different fractions were then subjected to nonlinear regression analysis, and RNA buoyancy peaks for each fungal species within a replicate were plotted. Peak fronts, e.g. the position of the heaviest RNA fraction of each of the AM fungal species detectable by qPCR, were calculated. Peak fronts further to the left indicate a higher ^{13}C enrichment in the fungal RNA (e.g. red peak front in the example shown). Peak front differences (delta values for RNA buoyancies in g ml^{-1} of each pair of AM fungal species within each replicate) were determined and provided a paired species comparison of the C allocation patterns.

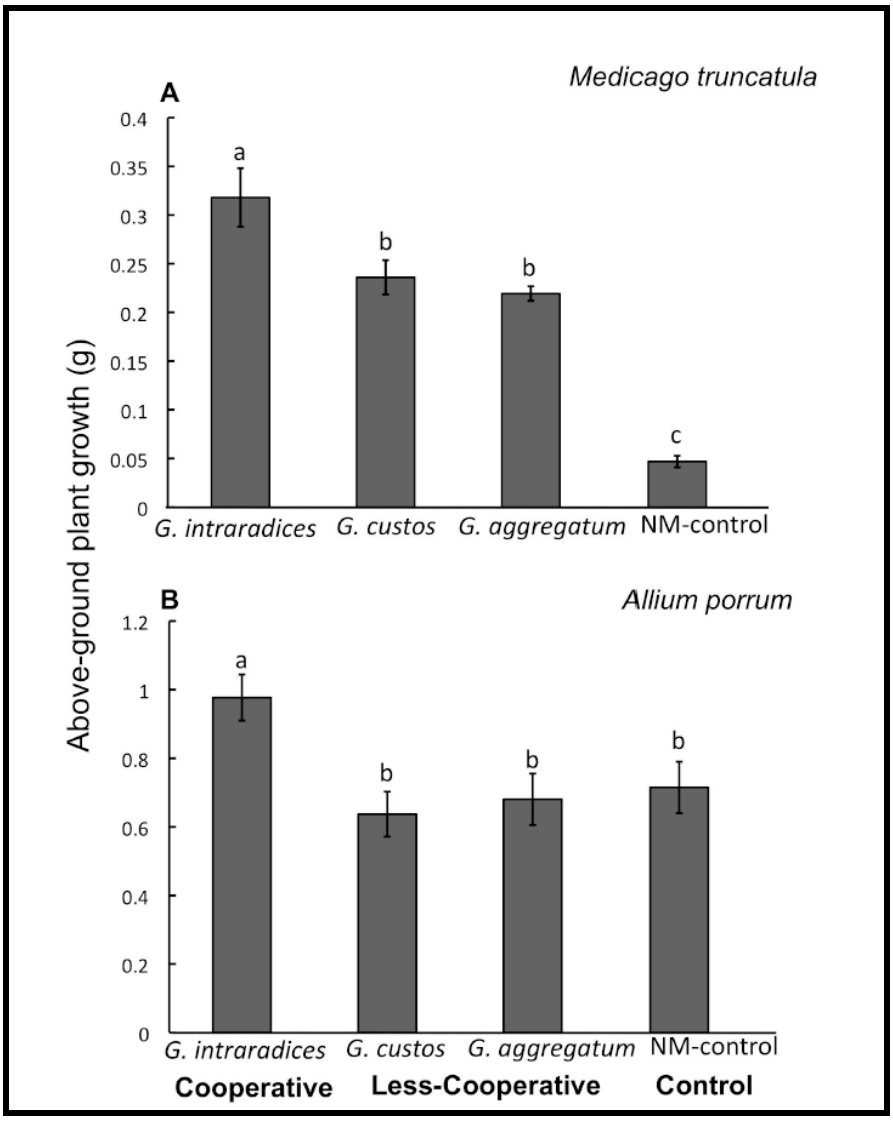

Fig. S3: Growth benefits conferred by the three AM fungal species and non-mycorrhizal (NM) controls. There was a significant effect of inoculation treatment in both the dicot and monocot plant species, (**A**) *Medicago truncatula* ($F_{3,65}$ = 52.808, P < 0.001) and (**B**) *Allium porrum* ($F_{3,58}$ = 4.494, P = 0.007). In *M. truncatula*, inoculation with the cooperative species (*G. intraradices*) led to a significant growth benefit compared to both less-cooperative species (*G. aggregatum* and *G. custos*) (Tukey's honestly significant difference (HSD), $P \leq 0.05$). These results were confirmed with the monocot *A. porrum*. *G. intraradices* again led to significantly higher growth than *G. aggregatum* or *G. custos* (Tukey's HSD, P ≤ 0.05). In both plant species, the less-cooperative strains were not 'parasites', meaning colonization by these fungal species lead to either greater (*M. truncatula*) or equal (*A. porrum*) growth compared to the NM-controls. This allowed us to examine whether hosts could detect and respond to variation in fungal cooperation, rather than testing for host response to a negative growth impact. Letters indicate significant differences between treatments means according to Tukey's HSD test ($P \leq$ 0.05). Bars represent the means of 15 replicates ± 1 standard error.

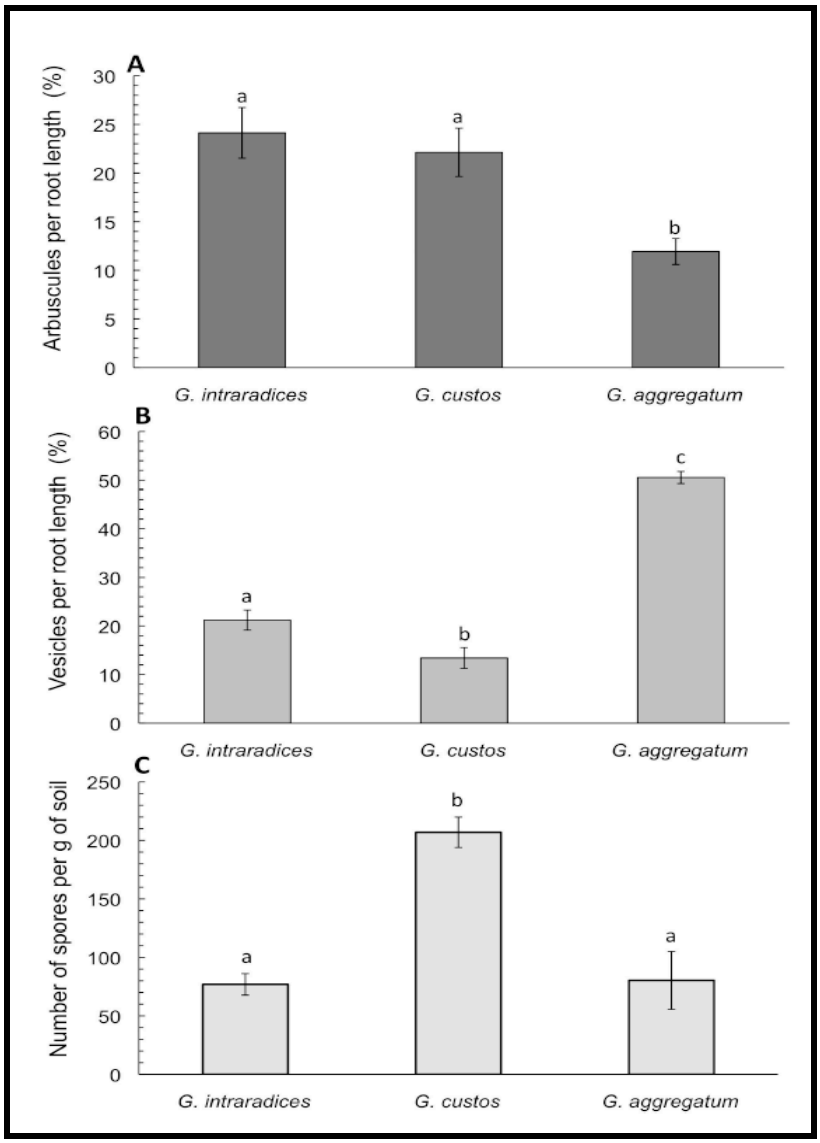

Fig. S4: Mycorrhizal growth characteristics of the three AM fungal species. All three species colonized more than 80% of the host root length of *M. truncatula* when grown alone, however structural patterns differed significantly among species. (**A**) The less-cooperative species *G. aggregatum* formed significantly less arbuscules per root length than the other two species ($F_{2,44}$ = 6.917, P = 0.003). (**B**) *G. aggregatum* formed significantly more vesicles per root length than the other two species ($F_{2,44}$ = 110.599, P <0.001). (**B**) The less-cooperative species *G. custos* invested significantly more in spores compared to the other two fungal species ($F_{2,26}$ = 18.747, P <0.001). Data were log transformed before analysis to meet assumptions for variance homogeneity. Different letters indicate significant differences between treatments means according to Tukey's HSD test ($P \leq 0.05$). Figures (**A**) and (**B**) show the means of 15 replicates ± 1 standard error. Figure (**C**) shows the mean of 9 replicates ± 1 standard error.

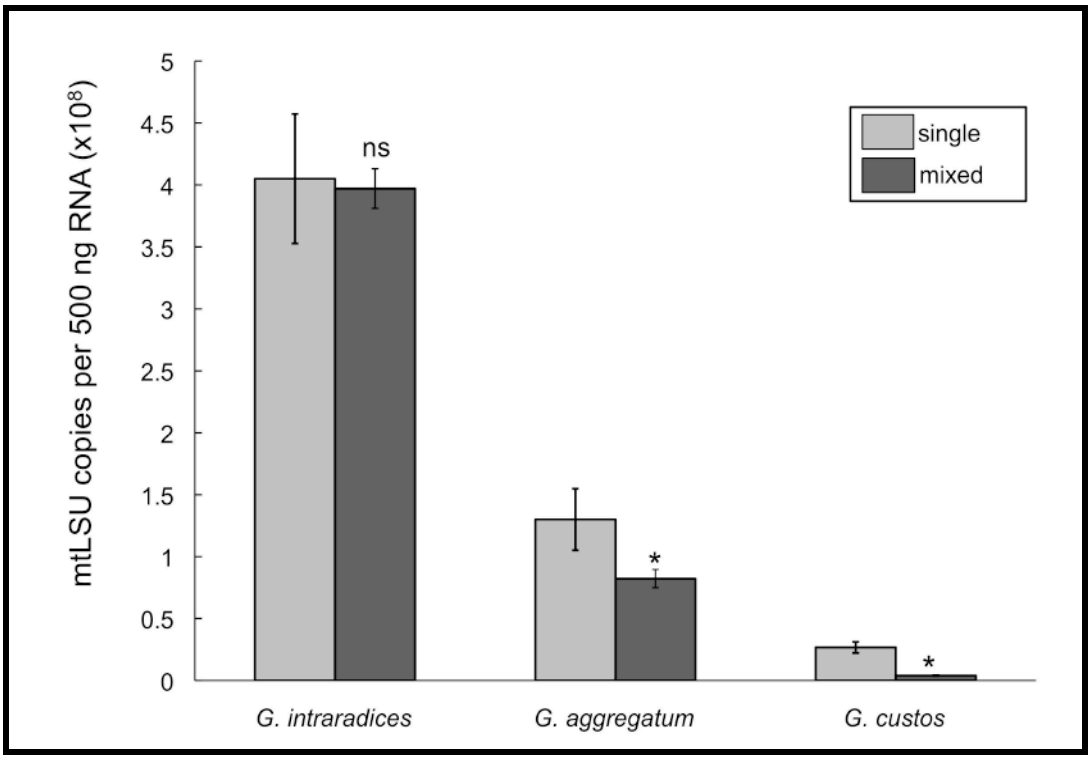

Fig. S5: Changes in the abundance of different AM fungal species in association with *M. truncatula*, when alone or in mixtures (e.g. equal proportions of all three species). Abundance of AM species was assessed by species-specific qPCR on cDNA prepared from non-fractionated RNA samples. There was no significant difference in the abundance of *G. intraradices* when the plant was inoculated with *G. intraradices* alone or in mixture ($F_{1,8}$ = 0.05, P = 0.84). In contrast, there was a significant decrease in the abundance of *G. aggregatum* ($F_{1,8}$ = 6.39, P = 0.035), and *G. custos* ($F_{1,8}$ = 63.6, P <0.001), when compared to their singly inoculated controls. Cochran`s C Test and Bartlett`s test indicated no major deviation from the null hypothesis of equal variance between treatments. Bars represent the means of n=3-7 ± 1 standard error. Asterisks indicate significant differences between treatment means.

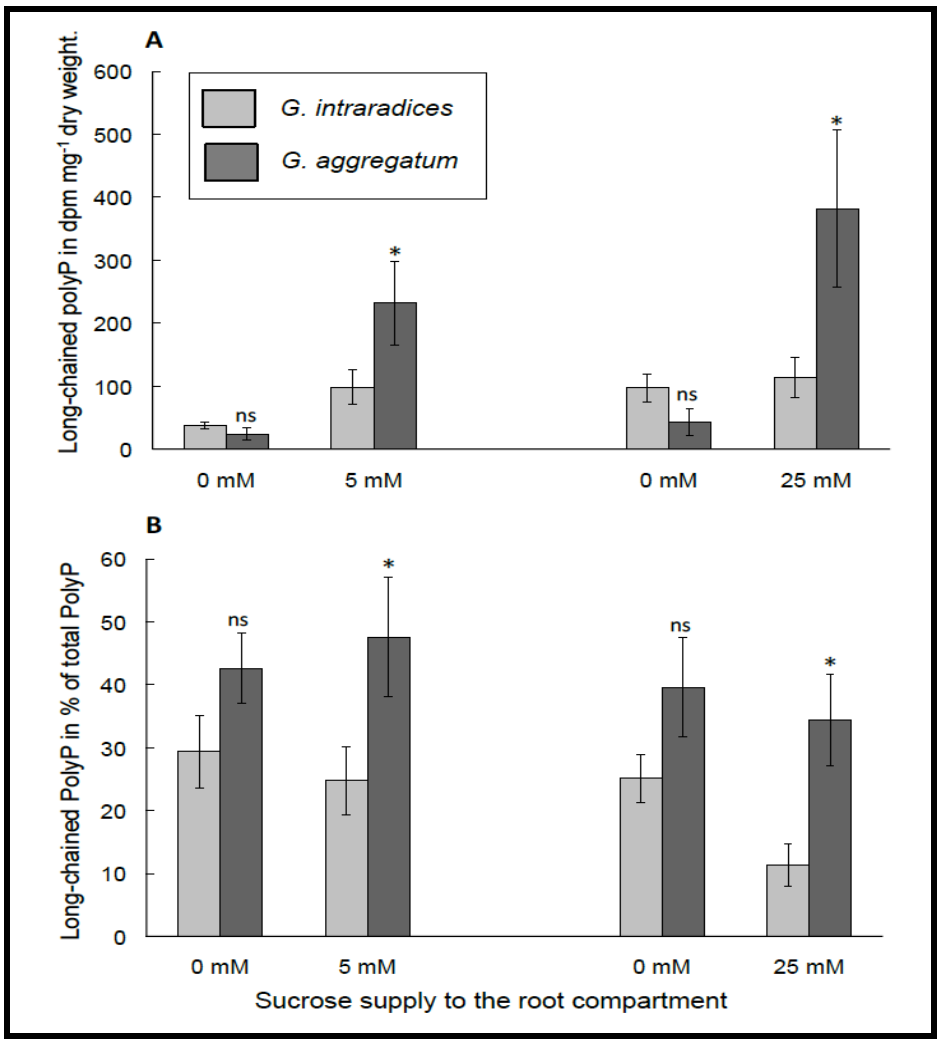

Fig. S6: Long-chain PolyP pools of cooperative and less-cooperative AM fungi in a one-fungal, two-root compartment experiment. The less-cooperative *G. aggregatum* transferred more P to the root system that was better supplied with C, but retained the P in the form of long-chained polyphosphates (PolyP) (Seufferheld & Curzi, 2010), a form unavailable for the host (Takanishi *et al.*, 2009). This could represent a potential hoarding strategy (see also Fig. S7, S8). In contrast, the cooperative fungus *G. intraradices* converted a larger proportion of its long-chained PolyP to short-chained PolyP. Short-chained PolyP are continuously broken down in the intraradical mycelium to orthophosphate, which is transferred across the mycorrhizal interface to the host plant, and represent the PolyP pool that is correlated to host plant benefit (Ohtomo & Saito, 2005). Long-chained PolyP concentrations were higher in roots that were colonized with the less-cooperative AM fungus *G. aggregatum* compared to roots colonized with *G. intraradices,* both in (**A**) dpm mg^{-1} root dry weight (5 mM $F_{1,13} = 4.42$; $P = 0.055$ and 25 mM $F_{1,15} = 6.10$; $P = 0.026$) and (**B**) in % of total polyP (5 mM $F_{1,14} = 10.051$; $P = 0.0068$ and 25 mM $F_{1,13} = 5.404$; $P = 0.0369$). The bars represent the mean of n= 6 to 9 replicates ± 1 standard error. Asterisks indicate significant differences between species within each sucrose treatment.

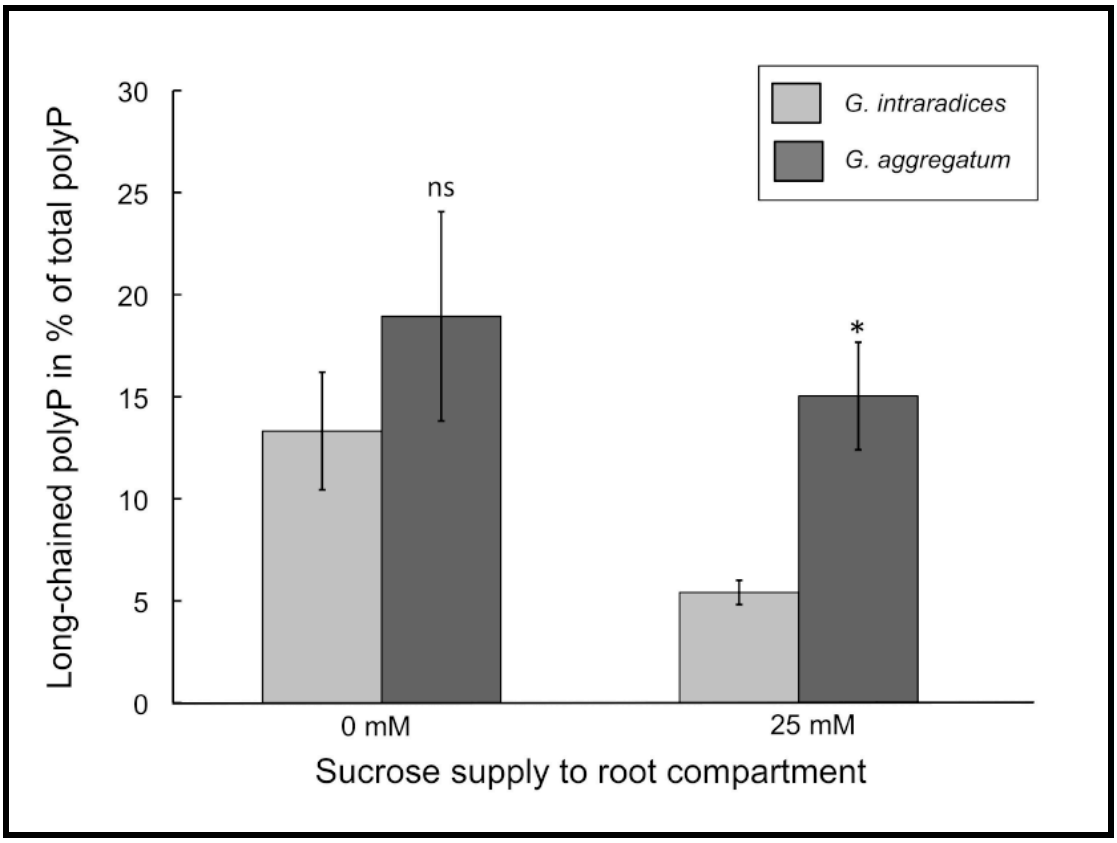

Fig. S7: The less-cooperative *G. aggregatum* retained significantly more P in form of long-chained polyphosphates (PolyP), than the cooperative AM fungus *G. intraradices*. As in the triple-plate experiment (Fig. S6), *G. aggregatum* retained the P in the form of long-chained PolyP. The differences were not significant when no sucrose was added to the root system (0 mM; $F_{1,13}$= 0.907, P = 0.341), but significant when 25 mM sucrose was added to the root system and more carbon became available for the fungus ($F_{1,8}$ = 12.682; P = 0.0074). The bars represent the mean of n= 5 or 8 replicates ± 1 standard error. Asterisks indicate significant differences between species within each sucrose treatment.

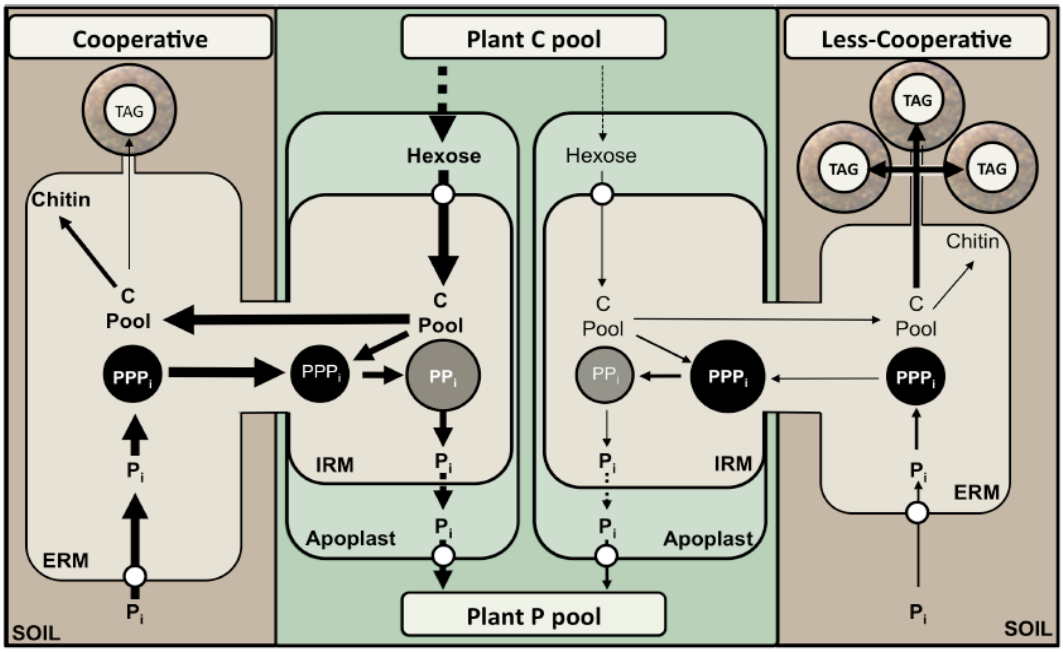

Fig. S8: Model showing carbon and phosphate exchange in roots colonized with a cooperative (left) or less-cooperative AM fungus (right). The host root allocates carbon preferentially to the cooperative AM fungus (Fig. 1), which invests C resources into structures for increasing nutrient uptake and exchange, such as chitin for the extension of the hyphae (e.g. extraradical mycelium, ERM) in the soil. This allows the cooperative AM fungus to absorb more inorganic orthophosphate (Pi) from the soil and to transfer more P to the host (Bücking & Shachar‐Hill, 2005; Lekberg *et al.*, 2010). The phosphate is transferred in the form of long-chained polyphosphates (PPPi, dark grey) to the intraradical mycelium (IRM) (Javot *et al.*, 2007). Here, the cooperative fungus breaks down long-chained polyP into short-chained polyP (PPi, light grey) (Figs. S6, S7) and then to inorganic orthophosphate (Pi). Short-chained polyP represents a relatively mobile polyP pool (Rasmussen *et al.*, 2000), while long-chained polyP represents a long-term storage pool of phosphate (Ohtomo & Saito, 2005; Takanishi *et al.*, 2009). This remobilization to short-chained polyP is likely facilitated by higher C conditions in the IRM (Bücking & Shachar‐Hill, 2005). The increase in the Pi pool in the IRM facilitates the efflux into the interfacial apoplast and the uptake by the plant from the apoplast via mycorrhiza-specific P transporters (Harrison *et al.*, 2002; Javot *et al.*, 2007). In contrast, the less-cooperative AM fungus invests more carbon resources, such as triacylglycerides (TAG) (Bago *et al.*, 2002) into the development of spores and vesicles (Fig. S4), and less into the development of nutrient absorbing ERM. Phosphate that is transferred to the IRM of the less-cooperative fungus is stored mainly in the form of long-chained polyP, and conversion to short-chained polyP is low (Figs. S6B, S7). This reduces the inorganic phosphate pool in the fungal cytoplasm and reduces the efflux of P through the fungal plasma membrane into the mycorrhizal interface that is driven by the concentration gradient between the fungus and the host (Smith *et al.*, 1994; Smith *et al.*, 1994; Ferrol *et al.*, 2002; Bücking & Shachar‐Hill, 2005). Storage of P in a long-chained form can be advantageous because it allows the fungus to better control the transfer of P across its plasma membrane by reducing P efflux. Hoarding of P resources also potentially reduces P availability for competing fungi and any P that is directly available for host uptake, making the host plant more dependent on the mycorrhizal pathway for its nutrients (Smith *et al.*, 2009; Smith & Smith, 2011). However, fungal P hoarding also results in higher carbon costs for P for the host when the plant is P deficient, and has no choice in fungal partners (Fig. 3). The different strength of the arrows indicates higher or preferential fluxes (bold) and lower or reduced fluxes (thin). Abbreviations: ERM - extraradical mycelium, IRM - intraradical mycelium, Pi – inorganic phosphate, PPi - short-chained polyphosphates, PPPi - long-chained polyphosphates, TAG – triacylglycerides.

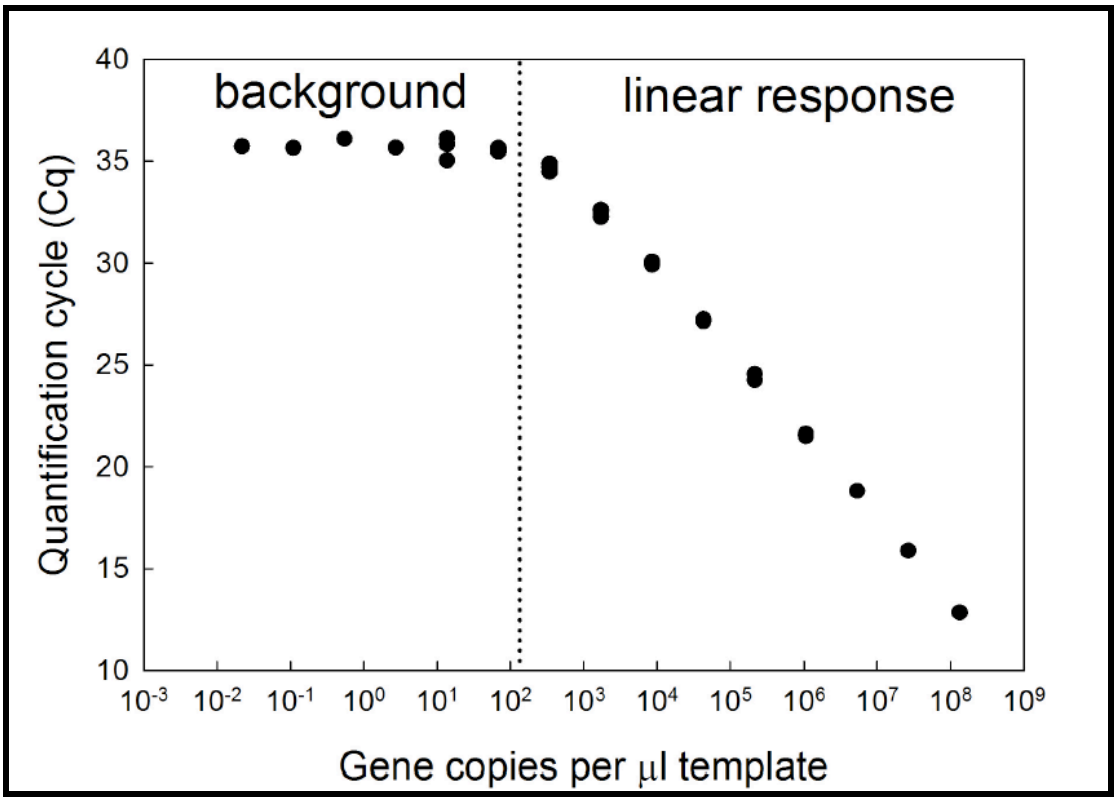

Fig. S9. Response of the qPCR signal (quantification cycle, Cq) to DNA template dilutions. Here, the intra mt5 marker for the DNA preparation of G. intraradices is shown. For the calibration of the qPCR assay only values of the linear response region were used. The background region was used to determine the detection limit of the qPCR assay.

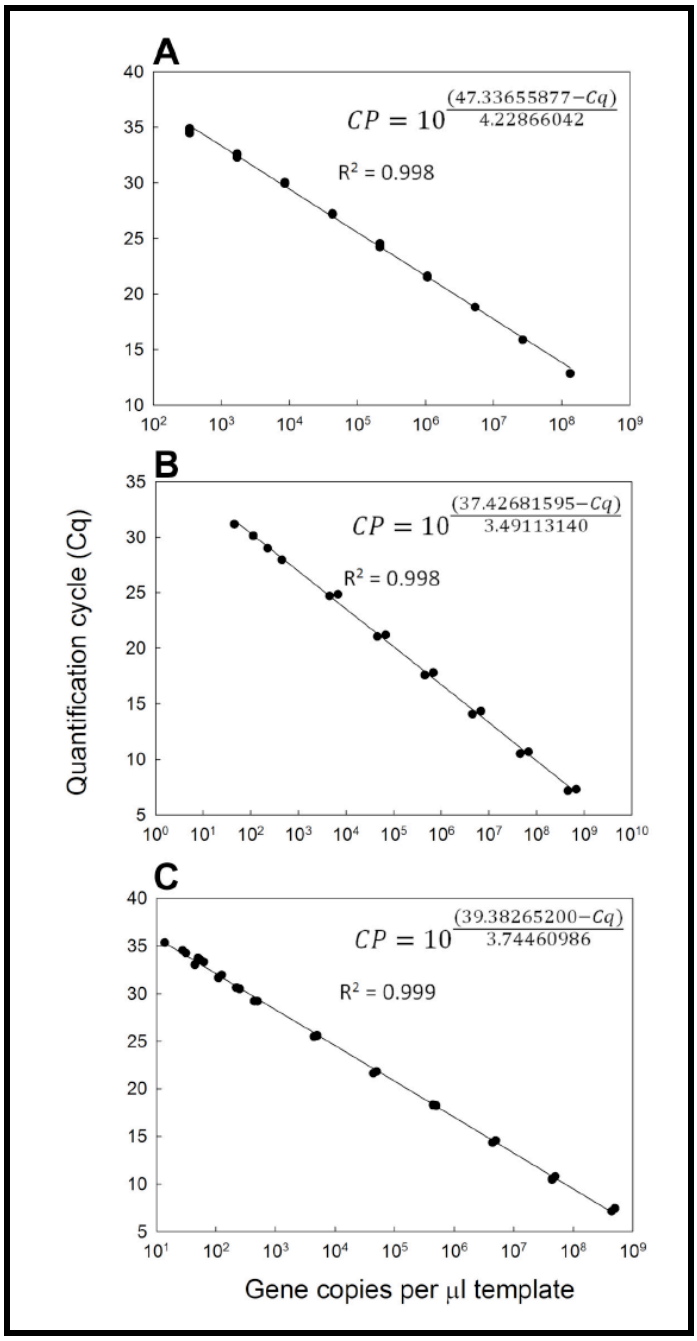

Fig. S10: Calibration curves for the qPCR assays. Curves were designed to assess abundance of AM fungal species with markers targeting species-specific sequence motifs of the mitochondrial large ribosomal subunit (mtLSU) of (**A**) *G. intraradices*, (**B**) *G. aggregatum* and (**C**) *G. custos*. The calibration was carried out with serially diluted plasmid preparations carrying the respective DNA fragments. Equations for the conversion of the qPCR signal (i.e., quantification cycle, Cq) to the gene copy concentrations in the template are given for each assay. CP represents the number of target gene copies per µl template.

2.5.5 TABLES

Table S1: qPCR markers for specific quantification of development of *Glomus intraradices, G. aggregatum*, and *G. custos* by measuring gene copies of the mitochondrial large ribosomal subunit of the respective AM fungal species. **FAM** – fluorescein, **BHQ1** – fluorescence quencher.

Target	**Sequences 5`→3` (forward primer, reverse primer, hydrolysis probe)**	**Nr cycles**	**Denaturation (°C / s)**	**Annealing (°C / s)**	**Amplification (°C / s)**
Glomus intraradices	TTTTAGCGATAGCGTAACAGC, TACATCTAGGACAGGGTTTCG, ***FAM***-AAACTGCCAC TCCCTCCATATCCAA-***BHQ1***	65	95 / 10	60 / 10	72 / 1
Glomus aggregatum	GGTATATTTCAAAGAGTAAGGTTCG, TGTCTCTACGCCTTAGTATGC, ***FAM***-AAAGAGCCCTA TGGAAACTTGCCTGAA-***BHQ1***	65	95 / 10	58 / 15	72 / 1
Glomus custos	TCTAACCCCAGAAATGTATAG, AAGGACTGCCTTGTGTTC, ***FAM***-ATACAATAATG GGCAATCAGACATATCGT-***BHQ1***	65	95 / 10	62 / 15	72 /1

Table S2: Results of cross-specificity assay under optimized (stringent) cycling conditions for each AM species-specific qPCR marker. For templates, we used DNA extracts from spores and roots, as well as cDNA preparations from root RNA extracts. Sample provenance gives the information where the sample was produced, not where the nucleic acids were extracted and/or processed. All the qPCR analyses were carried out in Eschikon, Switzerland, using the same Roche LightCycler 2.0 instrument and Roche TaqMan chemistry. ROC – root organ culture, nd – no signal detected, n.a. – not applicable, BLD – below detection limit of the particular marker system.

Sample description	Provenance	AM Fungus	Nature of template	Dilution before qPCR (fold)	Quantification cycle (Cq) with marker:		
					intra mt5	aggr	cust
Spores (ROC)	A. Bago	*G. intraradices*	DNA	10	31.78	nd	nd
Spores (ROC)	A. Bago	*G. custos*	DNA	10	nd	nd	27.56
Spores (ROC)	M. Hart	*G. aggregatum*	DNA	2	nd	25.74	nd
Roots (ROC)	M. Hart	*G. aggregatum*	DNA	2	nd	20.76	nd
Mycelium (ROC)	M. Hart	*G. aggregatum*	DNA	none	nd	26.74	nd
Roots (ROC)	M. Hart	*G. custos*	DNA	none	nd	nd	25.5
Roots (ROC)	M. Hart	*G. intraradices*	DNA	none	26.33	nd	nd
Roots (ROC)	H. Bücking via T. Kiers	*G. aggregatum*	DNA	none	nd	23.13	nd
Roots (ROC)	M. Hart	*G. custos*	DNA	100	nd	nd	29.15
Roots (ROC)	M. Hart	*G. intraradices*	DNA	100	31.66	nd	nd
Roots (ROC)	H. Bücking via T. Kiers	*G. aggregatum*	DNA	100	nd	29.98	nd
Root (pot culture)	O. Franken	*G. intraradices*	DNA	5	22.81	nd	nd
Root (pot culture)	O. Franken	*G. intraradices*	DNA	5	25.48	nd	nd
Root (pot culture)	O. Franken	*G. intraradices*	DNA	5	23.94	nd	nd
Root (pot culture)	O. Franken	*G. aggregatum*	DNA	5	nd	19.69	nd
Root (pot culture)	O. Franken	*G. aggregatum*	DNA	5	nd	19.03	nd
Root (pot culture)	O. Franken	*G. aggregatum*	DNA	5	nd	19.35	nd
Root (pot culture)	O. Franken	*G. custos*	DNA	5	nd	nd	22.68
Root (pot culture)	O. Franken	*G. custos*	DNA	5	nd	nd	23.03
Root (pot culture)	O. Franken	*G. custos*	DNA	5	nd	nd	23.33
Root (pot culture)	O. Franken	*G. intraradices*	DNA	5	23.48	nd	nd
Root (pot culture)	O. Franken	*G. intraradices*	DNA	5	27.13	nd	nd
Root (pot culture)	O. Franken	*G. intraradices*	DNA	5	24.85	nd	nd
Root (pot culture)	O. Franken	*G. aggregatum*	DNA	5	nd	22.56	nd
Root (pot culture)	O. Franken	*G. aggregatum*	DNA	5	nd	21.57	nd
Root (pot culture)	O. Franken	*G. aggregatum*	DNA	5	nd	19.46	nd
Root (pot culture)	M. Duhamel	non-mycorrhizal	cDNA	20	40.76 (BLD)	nd	nd
Root (pot culture)	M. Duhamel	non-mycorrhizal	cDNA	20	41.65 (BLD)	nd	nd
Root (pot culture)	M. Duhamel	non-mycorrhizal	cDNA	20	39.61 (BLD)	nd	nd
Root (pot culture)	M. Duhamel	*G. intraradices*	cDNA	20	34.74	nd	nd
Root (pot culture)	M. Duhamel	*G. intraradices*	cDNA	20	33.54	nd	nd
Root (pot culture)	M. Duhamel	*G. aggregatum*	cDNA	20	nd	29.17	nd
Root (pot culture)	M. Duhamel	*G. aggregatum*	cDNA	20	nd	28.54	nd
Root (pot culture)	M. Duhamel	*G. aggregatum*	cDNA	20	nd	27.96	nd
Root (pot culture)	M. Duhamel	*G. aggregatum*	cDNA	20	nd	28.14	nd
Root (pot culture)	M. Duhamel	*G. custos*	cDNA	20	nd	nd	30.97
Root (pot culture)	M. Duhamel	*G. custos*	cDNA	20	nd	nd	32.86
Root (pot culture)	M. Duhamel	*G. custos*	cDNA	20	nd	35.15 (BLD)	32.59

Continued Table S2.

Water (DNA, RNA, RNAase free)	Roche	n.a.	None	n.a.	39.93 (BLD)	39.33 (BLD)	nd
Water (DNA, RNA, RNAase free)	Roche	n.a.	None	n.a.	42.74 (BLD)	nd	nd
Water (DNA, RNA, RNAase free)	Roche	n.a.	None	n.a.	nd	nd	nd

Table S3: Detection limits and minimal detectable target gene concentrations of the three qPCR asssays.

AM fungal species (qPCR marker)	**Detection limit (detection cycle, Cq)**	**Threshold mtLSU gene copy concentration (copies μl^{-1})**
G. intraradices (intra mt5)	37.62	199
G. aggregatum (aggr)	30.52	95
G. custos (cust)	35.6	10

2.5.6 REFERENCES

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CHAPTER 3: NUTRIENT UPTAKE AND TRANSPORT: IMPLICATIONS FOR RESOURCE EXCHANGE IN THE ARBUSCULAR MYCORRHIZAL

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3.1 ABSTRACT

The arbuscular mycorrhizal (AM) symbiosis, which forms between plant hosts and ubiquitous soil fungi of the phylum Glomeromycota, plays a key role for the nutrient uptake of the majority of land plants, including many economically important crop species. AM fungi take up nutrients from the soil and exchange them for photosynthetically fixed carbon from the host. While our understanding of the exact mechanisms controlling carbon and nutrient exchange is still limited, we recently demonstrated that (i) carbon acts as an important trigger for fungal N uptake and transport, (ii) the fungus changes its strategy in response to an exogenous supply of carbon, and that (iii) both plants and fungi reciprocally reward resources to those partners providing more benefit. Here, we summarize recent research findings and discuss the implications of these results for fungal and plant control of resource exchange in the AM symbiosis.

3.2 INTRODUCTION

The AM symbiosis between fungi from the phylum Glomeromycota and the roots of approximately 65% of land plant species(Wang & Qiu, 2006) is characterized by an exchange of nutrients such as phosphorus (P) and nitrogen (N) from the fungus against carbon (C) from the host. AM fungi are obligate biotrophs and depend almost exclusively on host derived C to complete their life-cycle, and it has been estimated that the host transfers up to 20% of its photosynthetically fixed C to the fungus.(Wright *et al.*, 1998) This dependency of the fungus has led to the assumption that the host is in control of the

symbiosis, and that the nutrient transport in the mycorrhizal symbiosis is primarily driven by host plant demand.(Thomson *et al.*, 1990; Cairney & Smith, 1992; Cairney, 2011) In contrast, recent results indicate that, despite its high host dependency, the fungus can gain control in the symbiosis by adjusting its nutrient transfer in response to the C supply from the host(Bücking & Shachar-Hill, 2005; Hammer *et al.*, 2011; Fellbaum *et al.*, 2012). Both plants and fungi are able to detect variation in the resources supplied by their partners, allowing them to adjust their own resource allocation accordingly. These reciprocal reward mechanism ensures 'fair trade' between the symbiosis partners.(Kiers *et al.*, 2011) Here, we discuss these recent research findings in relation to strategies that both partners may use to regulate and maximize their nutritional benefit from the AM symbiosis.

3.3 CONTROL OF NUTRIENT UPTAKE PATHWAYS IN MYCORRHIZAL ROOTS

Mycorrhizal plants can acquire nutrients via two uptake pathways.(Smith & Smith, 2011) The direct pathway (DP) involves the uptake of nutrients from the soil-root interface by high affinity P and N transporters located in the root epidermis and its root hairs. The mycorrhizal pathway (MP) involves the uptake of nutrients from the fungal-soil interface by the extraradical mycelium (ERM), translocation to the intraradical mycelium (IRM) and uptake by the host from the fungal-plant interface (Fig.1) via mycorrhiza-inducible P and N transporters in the periarbuscular membrane.(Harrison *et al.*, 2002; Guether *et al.*, 2009)

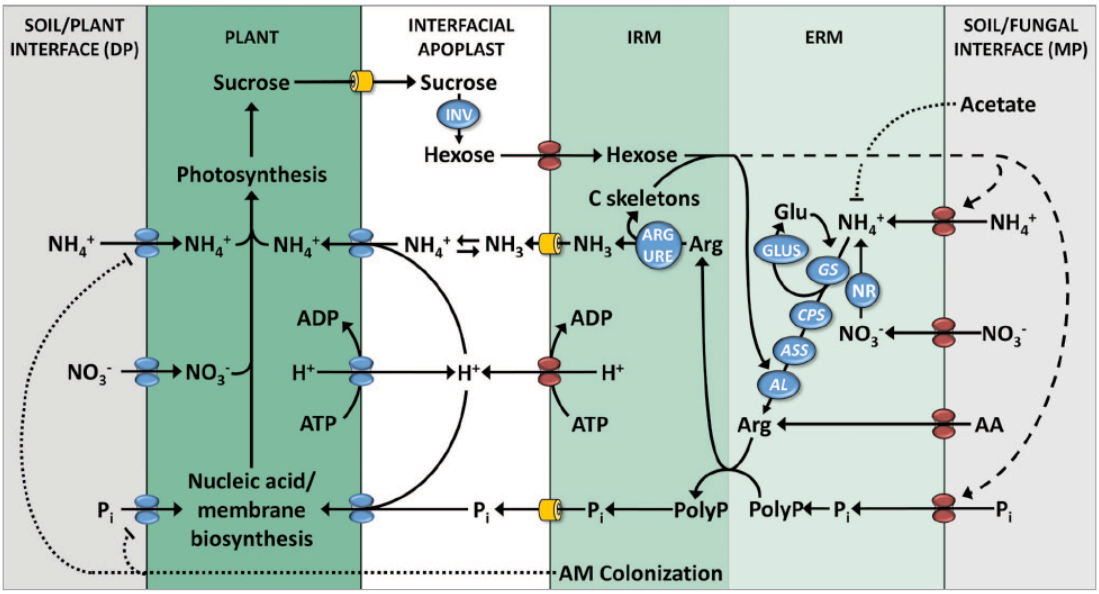

Fig. 1. Nutrient uptake via the direct pathway (DP) or mycorrhizal pathway (MP) in mycorrhizal roots. High affinity nutrient uptake transporters of the DP are downregulated in mycorrhizal roots (dotted line), and instead mycorrhiza-inducible transporters of the MP are expressed at the mycorrhizal interface. The ERM takes up inorganic N from the soil/fungal interface and N is assimilated and converted into arginine via glutamine synthetase (*GS*),carbamoyl-phosphate synthase glutamine chain (*CPS*), argininosuccinate synthase (*ASS*), and argininosuccinate lyase (*AL*). The basic amino acid arginine (Arg) acts as charge balance and is co-transported to the IRM with negatively charged polyphosphates (polyP) that are synthesized in the ERM from P taken up from the soil. PolyP are remobilized in the IRM and release inorganic P (P_I) and Arg, which is re-converted into NH_4^+ via the catabolic arm of the urea cycle and the activity of a fungal arginase (ARG) and urease (URE). Plants transfer sucrose into the interfacial apoplast, which is hydrolyzed by the activity of a plant invertase (INV) into hexoses. The carbon supply of the host stimulates N and P uptake and transport via the MP in the AM symbiosis (hatched line). The supply of a carbon source (acetate) independent from the C supply of the host reduces N transport in the AM symbiosis.

Plant uptake transporters of the DP are down-regulated in mycorrhizal roots,(Chiou *et al.*, 2001; Grunwald *et al.*, 2009) and the MP can represent the main uptake pathway even in plants in which no positive growth benefit is observed(Smith & Smith, 2011). Whether the suppression of the DP in mycorrhizal roots is a host driven or a fungal mediated response is not known. The expression of plant uptake transporters of the DP is normally regulated by host plant demand, and the lower transcript levels in mycorrhizal roots could only be the result of an improved P supply[13]. On the other hand, some transporters that are down-regulated in mycorrhizal roots are not controlled by P supply.(Liu *et al.*, 2008) It has been suggested that the suppression of the DP by AM fungi can even lead to growth depressions in mycorrhizal plants when the MP does not compensate for the reduced uptake of the DP. (Smith *et al.*, 2011) AM fungi differ in their efficiency with which they suppress the DP,(Grunwald *et al.*, 2009) and a strong suppression of the DP will shift the ratio between the two uptake pathways towards the MP and will result in a higher mycorrhizal dependency of the host. It is interesting to speculate that the AM fungus could use the down-regulation of the DP to increase its C availability. A higher dependence on the MP for nutrient uptake has been shown to stimulate the C allocation to the root system.(Nielsen *et al.*; Postma & Lynch, 2011)

3.4 CARBON AS TRIGGER FOR NUTRIENT UPTAKE AND TRANSPORT IN THE AM SYMBIOSIS

The host provides the fungus with C in the form of sucrose (Fig. 1), which is broken down by plant derived acid invertase(Schaarschmidt *et al.*, 2006; Schaarschmidt *et al.*, 2007) or sucrose synthase(Hohnjec *et al.*, 2003) into hexoses which the fungus takes up

via a high affinity monosaccharide transporter(Helber *et al.*, 2011). AM fungi are unable to use sucrose as a C source(Parrent *et al.*, 2009) and induce the expression of the plant acid invertase in the mycorrhizal interface(Schaarschmidt *et al.*, 2006). It has previously been shown that an increase in the C availability for the AM fungus stimulate the P transport in the AM symbiosis.(Bücking & Shachar-Hill, 2005; Hammer *et al.*, 2011) Our more recent work demonstrated that C also acts as trigger for fungal N uptake and transport, and that the stimulation in N transport is driven by changes in fungal gene expression (Fig. 1).(Fellbaum *et al.*, 2012)

Woolhouse in 1975 was the first to speculate that C and P transport in the AM symbiosis are directly linked,(Woolhouse, 1975) and this hypothesis was recently supported by the demonstration that the mycorrhiza-inducible plant P transporter *Pt4* and the fungal monosaccharide transporter *MST2* are co-localized in the AM interface and that their expression level is tightly linked.(Helber *et al.*, 2011) Phosphate transfer and the expression of *Pt4* is essential for the AM symbiosis; the absence of this transporter in the periarbuscular membrane leads to a premature degradation of arbuscules and the symbiosis fails.(Javot *et al.*, 2011) Arbuscules in the AM symbiosis undergo a cycle of growth, degradation, senescence and recurrent growth, and it has been suggested that a consistent host-driven turnover of arbuscules provides the plant with an instrument to remove and 'to penalize' inefficient AM fungal symbionts. This mechanism would also allow hosts to regulate its intracellular colonization according to changes in the exogenous nutrient supply conditions.(Breullin *et al.*, 2010) Interestingly, the arbuscular phenotype of *mtpt4* mutants is rescued by N deprivation, indicating that the AM fungus can escape arbuscular degradation by N transport across the mycorrhizal interface. Does

this mean that the host plant considers the sum of N and P benefits when regulating its intracellular colonization and the carbon supply to its fungal symbionts? More data are needed to answer this critical question.

3.5 NUTRIENT ALLOCATION IN COMMON MYCELIAL NETWORKS

AM fungi interact simultaneously within a common mycelial network (CMN) with multiple hosts from different plant species, and therefore do not rely on a single host for their C supply. Currently, it is not known how AM fungi allocate resources within a CMN and how host plants are able to compete with other plants for limited nutrient resources. It has been shown that carbon to nutrient exchange ratios in CMN are fungal and plant species-dependent and that plant species differ in their contribution to the C availability of the CMN. Recently, we demonstrated that AM fungi, despite the coenocytic nature of their hyphae, are able to distinguish between a C source that is supplied to the ERM or host C delivered via the mycorrhizal interface(Fellbaum *et al.*, 2012). When an exogenous supply of C became available for the AM fungus and the fungus became less dependent on its host for its C supply, a fungal arginase gene in the ERM was up-regulated, and the N transport to the mycorrhizal host was reduced. Consistently, a down-regulation of two fungal ammonium transporters was observed when an exogenous C source became available for the fungus(Pérez-Tienda *et al.*, 2011). This suggests that (i) there is a change in fungal strategy when the fungus has access to a C source independent from a single host and (ii) that the C supply of the host may also play an important role for the allocation of nutrients within a CMN. Recent work from

our lab in whole plant systems suggests that AM fungi allocate N and P resources in CMN according to the C benefit that different hosts are able to provide (Fellbaum et al., unpublished).

While significant progress has been made in understanding transport and allocation processes in the AM symbiosis, much more work is needed to understand the mechanistic strategies of both partners, and how these strategies are mediated by external resources. This will allow us to make predictions about mycorrhizal functioning under global change, and even allow us to maximize the benefits of the mutualism to increase the nutrient efficiency of crops in environmentally sustainable agriculture.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4: FUNGAL NUTRIENT ALLOCATION IN COMMON MYCORRHIZAL NETWORKS IS REGULATED BY THE CARBON SOURCE STRENGTH OF INDIVIDUAL HOST PLANTS

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4.1 SUMMARY

- Common mycorrhizal networks (CMN) of arbuscular mycorrhizal (AM) fungi in the soil simultaneously provide multiple host plants with nutrients, but the mechanisms by which the nutrient transport to individual host plants within one CMN are controlled, are unknown.
- Using radioactive and stable isotopes, we followed the transport of phosphate (P) and nitrogen (N) in the CMNs of two fungal species to plants that differed in their carbon source strength, and correlated the transport to the expression of mycorrhiza-inducible plant P (*MtPt4*) and ammonium (*1723.m00046*) transporters in mycorrhizal roots.
- AM fungi discriminated between host plants that shared a CMN and preferentially allocated nutrients to high quality (non-shaded) hosts. However, the fungus also supplied low quality (shaded) hosts with nutrients and maintained a high colonization rate in these plants. Fungal P transport was correlated to the expression of *MtPt4*. The expression of the putative ammonium transporter *1723.m00046* was dependent on the fungal nutrient supply and was induced when the CMN had access to N.
- Biological Market Theory has emerged as a tool to study the strategic investment of competing partners in trading networks. Our work demonstrates how fungal partners are able to retain bargaining power, despite being obligately dependent on their hosts.

4.2 INTRODUCTION

The 450-million year old arbuscular mycorrhizal (AM) symbiosis is among the world´s most widespread mutualisms and is formed by approximately 65% of all known land plant species (Wang & Qiu, 2006). The extraradical mycelium (ERM) of the fungus forms an extensive network in the soil and provides the host plant with access to nutrient resources beyond the root depletion zone. The ERM of the AM fungus takes up phosphate (P), nitrogen (N), sulfur and various trace elements from the soil, and transfers these nutrients to the intraradical mycelium (IRM), where the nutrients are exchanged for carbon from the host (Marschner & Dell, 1994; Smith *et al.*, 2009). The plant transfers up to 20 % of its photosynthetically fixed carbon to the AM fungus and the fungus uses these carbon resources to maintain and to extend its hyphal network in the soil (Wright *et al.*, 1998).

The maintenance of cooperation in the mycorrhizal partnership has long posed a paradox for evolutionary theory. Cooperation between different species is hard to explain because selfish individuals can exploit mutualisms, reaping benefits while paying no costs (Leigh, 2010). Sanctions - or other feedback mechanisms that allow a host to control the fitness of its partners - play a key role in stabilizing cooperation in many mutualisms (West *et al.*, 2007). However, in the mycorrhizal symbiosis, neither plant nor fungal partner is really ‘in control’. Both partners in the mycorrhizal symbiosis, interact with multiple partners simultaneously: a single plant host is colonized by multiple fungal species, and fungal ‘individuals’ interact with multiple plant hosts and species, interconnected by a common mycorrhizal network (CMN). This complex system of many-to-many

interactions means that neither partner can be 'enslaved', because both plant and fungus, can choose among multiple trading partners (Kiers *et al.*, 2011; Walder *et al.*, 2012).

Biological Market Theory is a useful framework to study how cooperation can be stabilized in many-to-many interactions. The theory argues that resource trade can be analyzed from an economic vantage point: partners on both sides of the interaction compete and those offering the best 'rate of exchange' will be favored (Noë & Hammerstein, 1995; Werner *et al.*, 2014). However for market dynamics to emerge, individuals must be able to discriminate among competing partners. Recently, a series of manipulative experiments demonstrated that mycorrhizal plants are able to detect, discriminate, and reward the best fungal partners with more carbohydrates (Kiers *et al.*, 2011). There is evidence that also fungal partners are able to discriminate and preferentially allocate P and N to roots grown under high carbohydrate conditions (Bücking & Shachar-Hill, 2005; Hammer *et al.*, 2011; Kiers *et al.*, 2011; Fellbaum *et al.*, 2012). However, these experiments have so far only been conducted in *in-vitro* root organ cultures, and it has been questioned whether these artificial systems function with enough ecological realism to capture the dynamics of the complex underground fungal networks that form among different plants in natural ecosystems (Smith & Smith, 2011).

CMNs can be formed by one individual fungus or when several conspecific fungal individuals connect by hyphal anastomoses (Mikkelsen *et al.*, 2008). In both cases, CMNs can transfer nutrients to several host plants simultaneously (van der Heijden & Horton, 2009; Lekberg *et al.*, 2010; Merrlid *et al.*, 2013). However, the mechanisms that determine how an AM fungus allocates nutrients among competing plants connected by one CMN are currently unknown. Recently, Walder *et al.* (2012) demonstrated that plant

species differ in their carbon investment into the CMN. They suggested that this contribution was unrelated to the amount of nutrients they receive. They found that the C3 plant flax, despite its smaller measured contribution to the carbon pool, still received the majority of nutrients from the CMN compared to the competing C4 plant sorghum. While this could be interpreted as evidence that AM fungi cannot discriminate among hosts of differing quality, this trade asymmetry can also be explained by differences in host-plant compatibility, carbon to nutrient exchange ratios, or other physiological differences (e.g. C3 vs. C4 photosynthesis). For example, sorghum showed lower levels of fungal colonization in roots and soil when grown in mixed cultures with flax, prohibiting standardized measurements of nutrient allocation to competing plants (Walder *et al.*, 2012).

To test for fungal discrimination processes, we varied photosynthetically active radiation by shading one of two *Medicago truncatula* plants and tracked fungal P and N allocation patterns of the CMNs of two fungal strains. We hypothesized that AM fungi could discriminate between high and low quality partners in a CMN and would preferentially transfer more N and P to high quality (non-shaded) partners. Considering the key role that AM fungi play for the nutrient uptake of land plants, plant community composition and carbon sequestration in ecosystems, it is crucial to better understand how carbon and nutrient resources are allocated in the CMNs of the most important and ancient symbiosis of land plants.

4.3 MATERIAL AND METHODS

Plant and fungal material

We scarified and germinated seeds of *Medicago truncatula* variety cv. Jemalong, A17 according to Salzer *et al*. (1999). After five days, we transferred two plants each into custom-made multi compartment systems filled with a sterilized (2 h at 121°C) growth substrate of 20% organic soil, 40% perlite, and 40% sand that contained 3.9 mg kg^{-1} NO_3, 40.3 mg kg^{-1} NH_4^+, and 1 mg kg^{-1} P (Olsen extraction) (v:v:v) (Fig. 1).

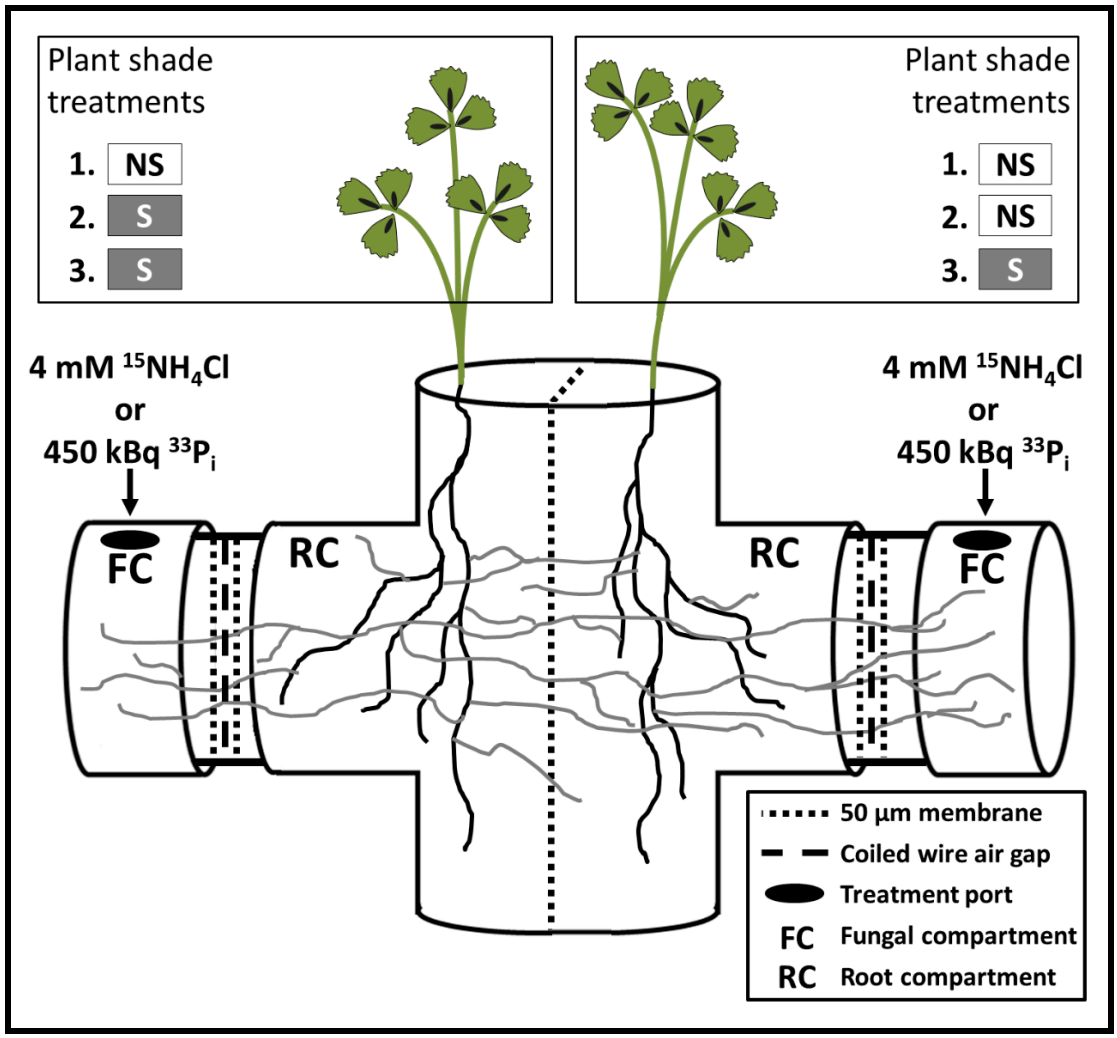

Fig. 1. Custom made growth system. A double membrane with an air gap (two sheets of 50 µm nylon mesh divided by a 30 cm long wire spiral) prevented the diffusion of nutrients from the fungal compartment (FC) to the root compartment (RC), but allowed fungal hyphae to cross from the RCs into the FCs. Three different shade treatments were applied to the plants: (1) both non-shaded (NS/NS), (2) one non-shaded, one shaded (NS/S), and (3) both shaded. To the FCs 4 mM $^{15}NH_4Cl$ or 450 kBq ^{33}P-orthophosphate was added.

These systems were constructed of a 3.81 cm (ID) PVC pipe and matching fittings. The root compartment was divided into two halves by a 50 µm nylon mesh (BioDesign Inc. of New York, USA) to prevent intermingling of roots, but allowed fungal crossover into both root compartments (RC). Fungal compartments (FC) were made out of a cap fitting joined by a 6 cm long PVC pipe, and separated from the RC by a double layer of a 50 µm nylon mesh, which was divided by a 30 cm long piece of wire (0.9 mm) wrapped into a spiral to prevent ion diffusion from the FC into the RC.

Twenty-four days after sowing, both plants were inoculated with 350 to 450 spores of either *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot; Walker & Schüßler, 2010; isolate 09 collected from Southwest Spain by Mycovitro S.L. Biotechnología ecológica, Granada, Spain) or *Glomus aggregatum* (N.C. Schenck & G.S. Sm.; isolate 0165 collected from the Long Term Mycorrhizal Research Site, University of Guelph, Canada). We selected these two fungal isolates because both fungi previously exhibited different levels of symbiont quality. Based on plant growth responses, and costs of carbon per unit P transferred, *R. irregularis* tends to be a more cooperative strain than *G. aggregatum* for *M. truncatula (Kiers et al., 2011)*. The inoculum was produced in axenic Ri T-DNA transformed carrot (*Daucus* carota clone DCI) root organ cultures in Petri dishes filled with mineral medium (St-Arnaud *et al.*, 1996). After eight weeks of growth, we isolated the spores by blending the medium in 10 mM citrate buffer (pH 6.0).

The plants were grown in a growth chamber (Conviron model TC30 Winnipeg, Canada) under the following conditions: 14 h photoperiod, 25°C/20°C day/night cycle, photosynthetically active radiation of 225 µmol m^{-2} s^{-1}, and 30% humidity. We watered the RC with 40 ml distilled water every four days, and the FC when needed. The plants

were fertilized once halfway through the growing period by adding a modified Ingestad solution with 250 µM NH_4NO_3 and 100 µM KH_2PO_4 to the RC of each growth chamber (Ingestad, 1960). The P and N concentration of the fertilizer was reduced to maintain a low nutrient status and a high mycorrhizal colonization rate of the plants. The plants exhibited signs of nutrient stress such as stunted growth and yellowish leaves at the time of P and N labeling.

Experimental Design

We conducted two experiments (N or P addition) to test the effect of carbon availability on resource allocation, and reduced the photosynthetically active radiation by applying a shade treatment to neither (non-shaded/non-shaded; NS/NS), one (non-shaded, shaded; NS/S) or both plants (shaded/shaded; S/S) in each growth system by covering the entire plant with a sheath made out of 12 cm x 14 cm 50% black shade cloth (Growers Solution Tennessee, USA). The shading reduced the photosynthetically active radiation by 60% from 222.75 µmol m^{-2} s^{-1} to 89.1 µmol m^{-2} s^{-1} as measured with a Li-Cor LI-185b light meter (Li-Cor, Lincoln, NE). One day after the plants were shaded, we injected 17.1 mg (leading to a concentration of 4 mM) 99% enriched $^{15}NH_4Cl$ (Cambridge Isotope Laboratories, Tewksbury, MA) dissolved in 6 ml, or 450 kBq (0.078 ng) ^{33}P-orthophosphate (Perkin Elmer, Waltham, MA) dissolved in 3 ml purified H_2O through a port to the FCs. Previous tests indicated that these labeling volumes homogenously wet the substrate in the FC without saturation. There were five biological replicates per shade treatment and nutrient supply and each treatment was inoculated with either *R. irregularis* or *G. aggregatum*. In total, 30 systems each were supplied with N or with P. Additionally, three systems each with non-inoculated controls were labeled to

confirm that there was no significant leakage of $^{15}NH_4^+$ or of ^{33}P from the FC into the RC.

Since the detectability of the radioactive label is higher but time sensitive (^{33}P half-life is 25.3 d), we harvested the plants 5 days after P addition. Since the ^{15}N analysis is not as sensitive, and in preliminary experiments the ^{15}N labeling of the plants was too low after 14 d, the plants were harvested 23 days after N was supplied to the FC. At harvest, the roots were cleaned and the total fresh weight was taken. The roots were then divided into three aliquots, the first aliquot was weighed, dried at 70°C for 2 days and the dry to fresh weight ratio was used to determine the total root dry weight. This aliquot was later used for ^{33}P and ^{15}N analysis. The second aliquot was cryofixed in liquid nitrogen and stored at -80°C for RNA extraction, and the third aliquot was stored in 50% ethanol until the mycorrhizal colonization assays were performed. The shoots were stored at -80°C until they were ground in a mortar cooled with liquid nitrogen, lyophilized, weighed and prepared for elemental analysis.

Analysis of mycorrhizal colonization and ERM development

We extracted ERM from the FC as described previously (Miller *et al.*, 1995) with slight modifications. The ERM was collected with a 50 µm nylon mesh (BioDesign Inc. of New York, USA) and stained for 30 min. The nylon mesh with the ERM was rinsed with 2 ml Milli Q and collected on a 0.45 µm gridded membrane (Millipore, USA), air dried and mounted with 30% glycerol. The length of the ERM was quantified according to Brundrett *et al.* (1994). We also determined the percentage of the total root length

colonized in a minimum of 50 root segments by the grid intersection technique (McGonigle *et al.*, 1990).

Analysis of ^{33}P labeling and extraction of various P pools

We analysed the soil of the RC to make sure that there was no diffusion of nutrients from the FC into the RC. Aliquots of the soil were dried at 70°C, and the P content was extracted with 0.5 M $NaHCO_3$ at pH 8.5 according to Olsen *et al.* (1954). The samples were vortexed, allowed to sit for 30 min and then centrifuged. An aliquot of the supernatant was taken and the ^{33}P content was measured by liquid scintillation counting. After homogenization of the root and shoot samples, an aliquot was taken, dried at 70°C, weighed and digested by adding 500 µl tissue solubilizer to the sample (TS-2, Research Product international, Mount Prospect, IL). After digestion, 150 µl glacial acetic acid and 2 ml scintillation cocktail (Biosafe II; Research Product international) was added. We also analyzed in mycorrhizal root samples the allocation of P into different P pools according to Aitchison and Butt (1973). The samples were dried at 70°C, homogenized and the following P pools were analysed: ortho-phosphate (P_i) and acid soluble or short-chained polyphosphates (polyP) with a chain length of ≤ 20 phosphate residues after extraction with ice-cold 10 % TCA; phospholipids after extraction with 100 % ethanol and ethanol/ether (3:1, v:v); acid-insoluble polyP with a chain length of > 20 phosphate residues after extraction with 1 M KOH; and DNA-, RNA-, and protein-phosphates as residue after extraction of all other pools. All samples were measured with a Wallac

scintillation counter (Perkin Elmer, Waltham, MA) and the data were corrected for differences in the counting efficiency by use of an internal standard.

Analysis of ^{15}N labeling

We digested 10-15 mg aliquots of homogenized and freeze-dried root and shoot material in 750 µl concentrated H_2SO_4 and heated the samples for 2 h at 225°C followed by an addition of 36 drops of 30% H_2O_2 (three drops at a time every 30 sec) as described earlier (Fellbaum *et al.*, 2012). The solution was then heated for an additional 3 h at 225°C to remove any traces of water and allowed to cool. Forty microliter of the resulting clear solution of $(NH_4)_2SO_4$ in H_2SO_4 with dissolved into 600 µl of 99.9 % d_6 DMSO containing 0.05% (v:v) TMS reference. The ^1H spectrum was obtained in a 5 mm z-axis PFG dual broad-band probe on a 9.2 Tesla Varian Inova spectrometer operating at 400 MHz. The spectra were acquired using ~1400 transients with a 90° (10.8 µsec) pulse width, spectral width of 5042 Hz, pulse delay of 1.0 sec, acquisition time of 1.6 sec at 25°C. The T_1 relaxation time of the NH_4 protons were measured to be 0.4 sec. The triplet resonance of the ^1H-^{14}N and doublet resonance of the ^1H-^{15}N were observed centered at 7.2 ppm relative to the TMS resonance 0.0 ppm with observed ^1H-^{15}N couplings of 53 Hz and 74 Hz, respectively. The percentage of total N labeled with ^{15}N in the tissue was determined by dividing the integrated area of the ^1H-^{15}N doublet resonances by the sum of the integrated doublet and triplet resonance areas (Fig. S1).

Quantitative real-time PCR of genes involved in nitrogen and phosphate transport

Using quantitative real-time PCR (qPCR), we studied the transcript levels of genes encoding the mycorrhiza-inducible plant P transporter *MtPt4* (Chiou *et al.*, 2001; Harrison *et al.*, 2002; Javot *et al.*, 2007), and of *1723.m00046*, a mycorrhiza-inducible plant ammonium transporter that is induced in cortical cells harboring arbuscules (Gomez *et al.*, 2009). All steps were performed according to the manufacturer's instructions unless otherwise stated. We homogenized the root samples with a mortar and pestle cooled with liquid nitrogen, and extracted total RNA using TRIzol Reagent (Invitrogen, Grand Island, NY). The supernatant was treated with an RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA) and the RNA was eluted into 1 µl of an RNase inhibitor (Murine, New England Biolabs, Ipswich, MA). The extracted RNA was treated using RQ1 RNase-Free DNase (Promega, Madison, WI) and quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized using 0.15 µg μl^{-1} DNase treated RNA, MMLV Reverse-Transcriptase (Promega), Random Primer 6 (New England Biolabs), and dNTPs (Qiagen). qPCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen), 2 µl of 1:5 diluted cDNA, 0.625 µM forward and reverse primers (NCBI: *MtPt4*: Pr010288303; *1723.m00046*: Pr010288319; *EF1α*: Pr010288292; Gomez et al., 2009) in a 20 µl reaction using an ABI 7900HT thermal cycler (Applied Biosystems, Grand Island, NY). The PCR conditions were as follows: 56°C for 2 min; 95°C for 15 min; 40 cycles at 95°C for 10 s, 60°C for 15 s, and 72°C for 10 s; dissociation at 95°C for 15 s; 60°C for 15 s; and 95°C for 15 s. Changes in gene expression (*MtPt4*: NCBI AY116211; *1723.m00046)* were compared to non-mycorrhizal control plants and by using *EF1α* (TC106485) as a reference gene

(Gomez *et al.*, 2009) and the ΔΔCT method (Winer *et al.*, 1999). The results are based on 3-12 biological replicates and 2 technical replicates.

Statistical treatment

If not mentioned otherwise, we only discuss treatment effects when they were statistically significant according to two-way ANOVA with inoculation (*G. aggregatum* or *R. intraradices*), or shade treatment (various shade treatments) or three-way ANOVA with inoculation (*G. aggregatum* or *R. intraradices*), shade treatment (various shade treatments), and nutrient supply to FC (^{15}N or ^{33}P) as factors. Paired t-tests were used to compare shaded and non-shaded plants in NS/S systems and treatment effects were tested by Fisher´s Least Significant Difference (LSD) test ($p \leq 0.05$). If the within treatment variability was too high, we log transformed the data prior to analysis. If a normal distribution of the data could not be guaranteed, we used the non-parametric Mann-Whitney´s u-test (given in the text). Correlations and computed *p* values were analyzed by Pearson´s correlation coefficient. All tests were conducted with JMP 10 (Cary, NC) or Unistat 6.0 (London, U.K.).

4.4 RESULTS

Host plant growth and mycorrhizal colonization by fungal partners

First, we analyzed host plant biomass to determine the effect of shading and fungal inoculation (Fig. S2). There was a high variability in the root and shoot biomass of all treatments and even though some statistical differences were observed (Fig. S2a, d),

there were no consistent effects related to shading or fungal inoculation. The analysis of the hyphal length in each FC demonstrated that in growth systems with one shaded and one non-shaded plant (NS/S) the ERM development did not differ among the two FCs, and that both fungal species successfully established a CMN in the growth systems (Table S1). While the hyphal lengths of *G. aggregatum* were slightly higher (78.4 ± 17.8 m g^{-1} dry) than of *R. irregularis* (53.8 ± 4.4 m g^{-1} dry soil), there was no statistically significant difference between both fungal species (Table S1). However, consistent with the slightly higher ERM development, we found a higher labeling with ^{33}P or ^{15}N in roots colonized with *G. aggregatum* (see below, Table S2, S3). Shading had a significant effect on the mycorrhizal colonization. While the colonization levels of both fungi were high for NS/NS and NS/S systems (> 93.8 %), these levels decreased in S/S systems to 73.7 % for *G. aggregatum* and 77.5 % for *R. irregularis* (Fig. S3).

Phosphate and nitrogen allocation in common mycorrhizal networks

When the fungus had access to a shaded and a non-shaded host plant, both fungi preferentially transferred more of the P and N taken up to non-shaded hosts (Fig. 2, Table S2, S3). This suggests that both fungal strains were able to discriminate between host plants, and preferentially allocated resources to non-shaded plants.

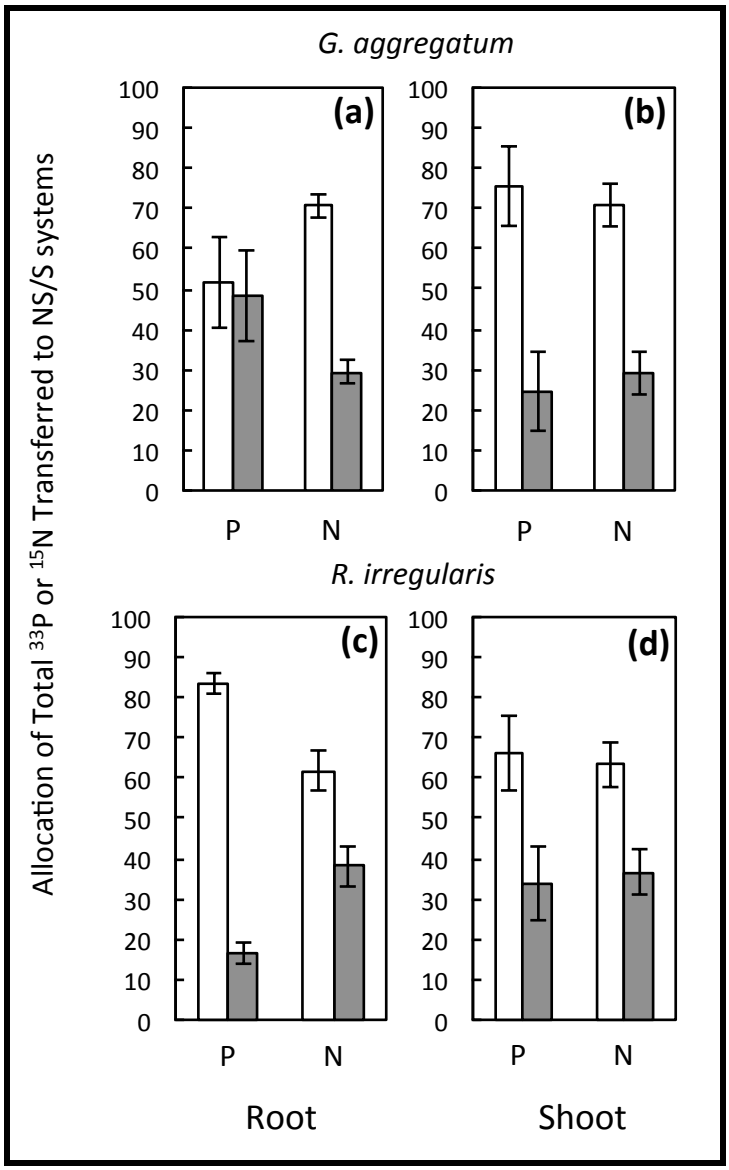

Fig. 2. Allocation of the total ^{33}P or ^{15}N taken up by the CMN of *G. aggregatum* (a, b) and *R. irregularis* (c, d) and transferred to the roots (a, c) or shoots (b, d) of non-shaded (white bars) and shaded (grey bars) plants in NS/S systems (calculated as percent based on the N or P content in shaded and non-shaded plants). Shown is the average of n=5 ± SE.

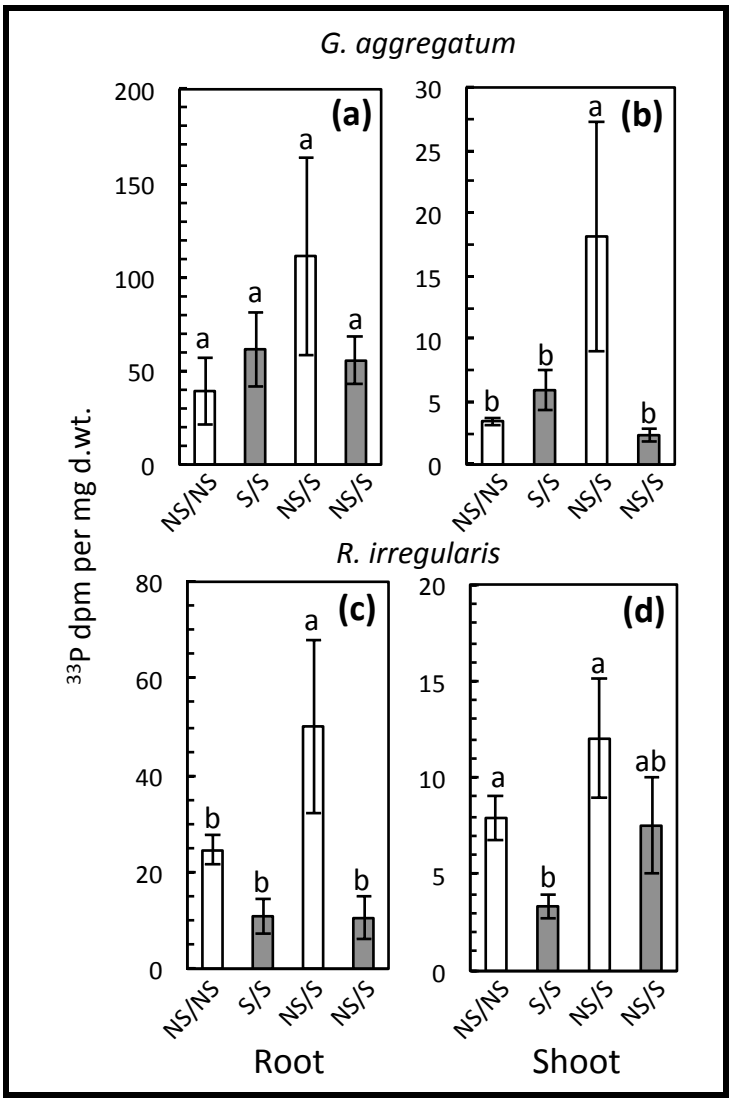

Fig. 3. Phosphate transport (in dpm mg^{-1} d.wt.) of the CMN of *G. aggregatum* (a, b) and *R. irregularis* (c, d) from the fungal compartment to root (a, c) or shoot (b, d) of the host plants dependent on the photosynthetic capability (non-shaded, white bars, and shaded, gray bars). Systems with two non-shaded (NS/NS), two shaded (S/S) or one non-shaded and one shaded plant (NS/S). Shown is the average of n=5-12± SE. Different letters on the bars indicate statistically significant differences within each graph according to LSD test ($p \leq 0.05$). The results of the two-way ANOVA are shown in Table S2.

This preferential allocation by *G. aggregatum* led to significantly higher P levels in the shoots (but not roots) of non-shaded hosts of NS/S systems (Fig. 3a, b). In growth systems with a shaded and a non-shaded plant (NS/S), *G. aggregatum* transferred more P to the shoots of non-shaded plants, but less P to the shoots of shaded plants, compared to systems in which the fungus had only access to shaded or to non-shaded plants (S/S or NS/NS; significant according to u-test, $p = 0.0152$) (Fig. 3b). In *R. irregularis* inoculated plants, the preferential allocation resulted in higher P levels in both the roots and shoots of non-shaded plants, independent of whether systems in which both plants were shaded or non-shaded (S/S vs. NS/NS) were compared, or systems in which the fungus had access to both a shaded and a non-shaded plant (NS/S; Fig. 3c,d).

Between 5.5 to 17.3 % of the total P transferred to the mycorrhizal roots was stored in form of long-chained or short-chained polyphosphates (poly-P). Fungal strain and shading did not have a significant effect on the total poly-P content, and in roots colonized with *G. aggregatum*, the proportion of long-chained to short-chained poly-P was unaffected by the shading treatment. However, we observed a shift in the ratio between long-chained to short-chained poly-P in roots that were colonized with *R. irregularis*: a significantly higher proportion of the poly-P in the roots of shaded plants was stored in the form of long-chained poly-P (Fig. S4).

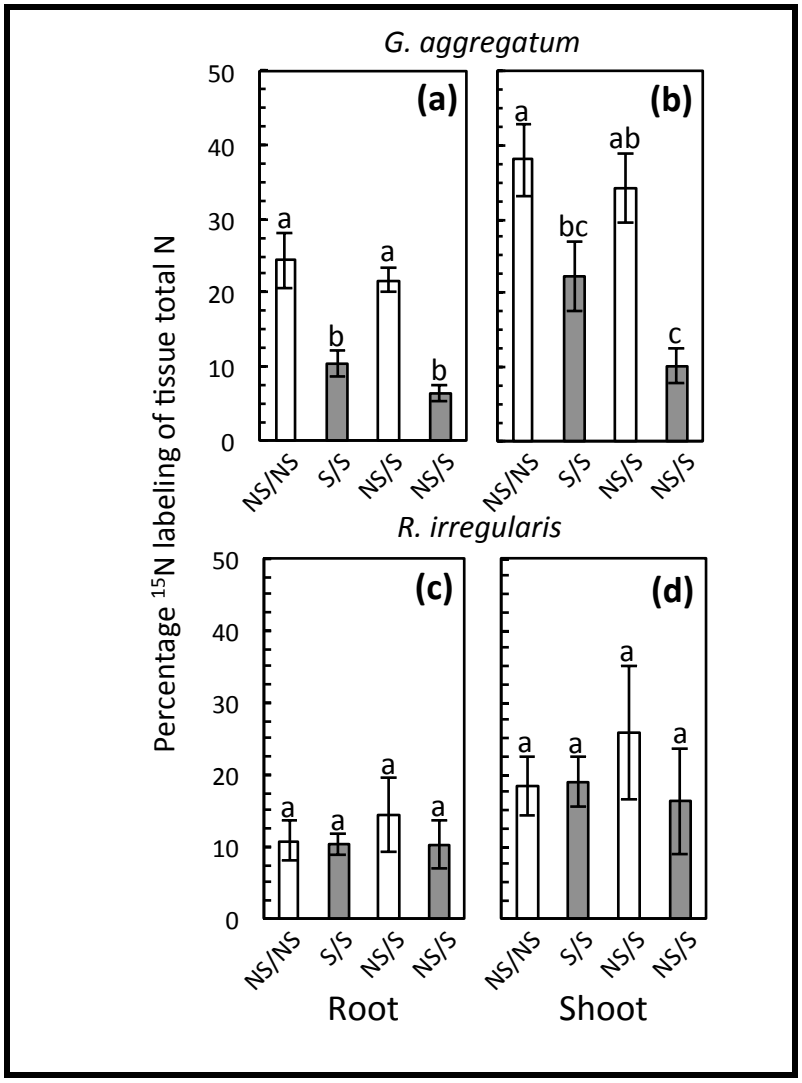

Fig. 4. Nitrogen transport (in percent ^{15}N labeling of total N) of the CMN of *G. aggregatum* (a, b) or *R. irregularis* (c, d) from the fungal compartment to root (a,c) or shoot (b,d) of the host plants dependent on the photosynthetic capability (non-shaded, white bars, and shaded, gray bars). Systems with two non-shaded (NS/NS), two shaded (S/S) or one non-shaded and one shaded plant (NS/S). Shown is the average of n=5-8 ± SM. Different letters on the bars indicate statistically significant differences within each graph according to LSD test ($p \leq 0.05$). The results of the two-way ANOVA are shown in Table S3.

Both fungi transferred N from the fungal compartment to their host and up to 24.4 % of the N in the root and up to 38.0 % of the N in the shoot became labeled with ^{15}N (Fig. 4a, b; Fig. S1). The shading had a significant effect on the N labeling in roots and shoots of *G. aggregatum* but not in *R. irregularis* (Fig. 4). The CMN of *G. aggregatum* transferred significantly more N to roots and shoots of non-shaded host plants (Fig. 4a, b). When shaded plants in NS/S or S/S systems were compared, *G. aggregatum* transferred more N to the shoots of shaded plants when only shaded plants were available as hosts (S/S); the difference in the ^{15}N labeling on a dry weight basis was not significant on the 5 % level ($p = 0.0743$) (Fig. 4b). There was a high variability in the ^{15}N labeling in the plants that were colonized with *R. irregularis*, and shading did not lead to a significant reduction in the ^{15}N labeling of the plants (Fig. 4c, d).

Expression of plant P and N transporters in mycorrhizal roots

MtPt4 expression was induced in mycorrhizal roots colonized by both fungi. However, the fold induction depended on the fungal species colonizing the root; while *G. aggregatum* induced the expression of *MtPt4* up to 90-fold compared to the non-mycorrhizal controls (particularly under non-shaded conditions), we only found inductions up to 25-fold in roots colonized with *R. irregularis* (Fig. 5).

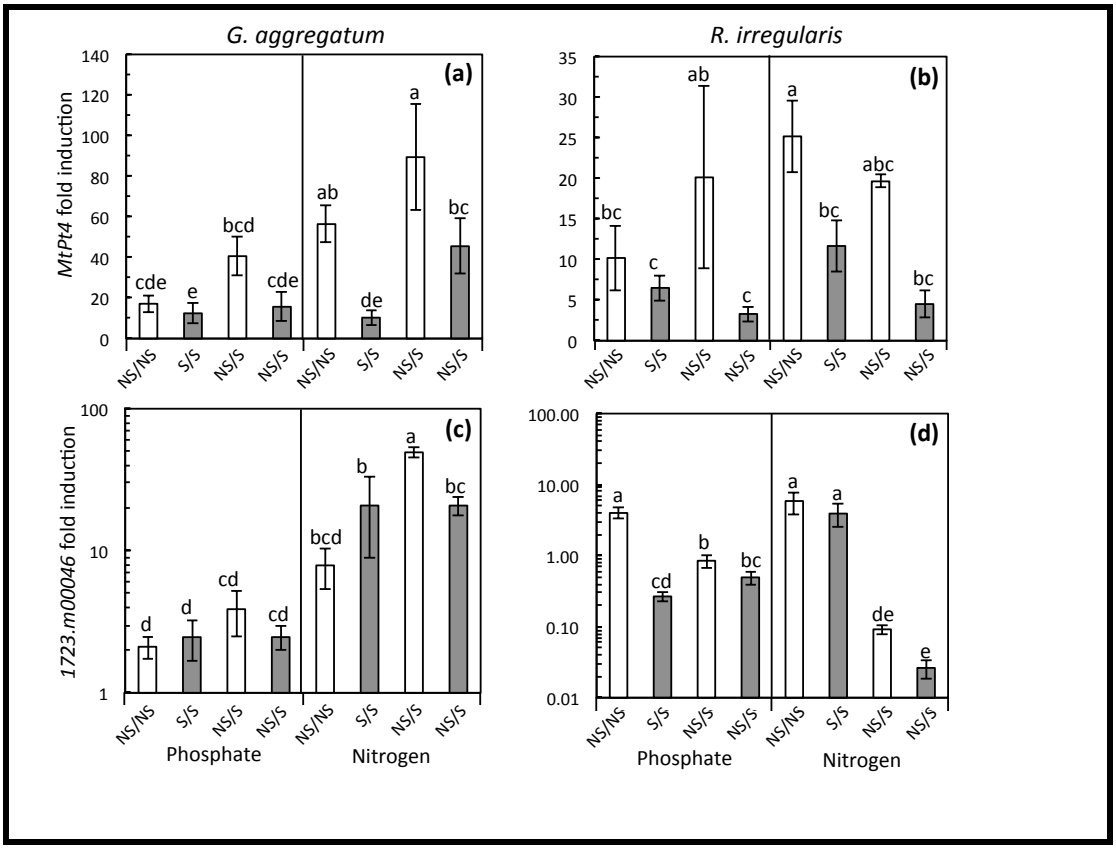

Fig. 5. Expression of *MtPt4* (a, b) and *1723.m00046*m (c, d) in roots that were colonized by *G. aggregatum* (a, c) or *R. irregularis* (b, d). Systems with two non-shaded plants (NS/NS), two shaded plants (S/S), or one non-shaded and one shaded plant (NS/S); non-shaded plants (white bars) and shaded plants (grey bars), or systems to which ^{33}P (phosphate) or ^{15}N (nitrogen) was added to the fungal compartments. Shown is the average of n=3-12± SE. Different letters on the bars indicate statistically significant differences according to LSD test ($p \leq 0.05$). Results of a three-way ANOVA are shown in Table S4.

Consistent to the reduction in the P transport to the shaded hosts, we found that shading reduced the *MtPt4* expression in mycorrhizal roots (Fig. 5a, b) independently on whether the fungus had access to both non-shaded and shaded plants or only to shaded or non-shaded plants. The decrease in the *MtPt4* transcript levels in shaded plants was particularly pronounced in systems colonized by *R. irregularis*; the transcript levels of the non-shaded plants was four to five times higher than of the shaded plants in NS/S systems. The expression of *MtPt4* was proportional to the P transport to the colonized roots and was correlated to the P tissue concentration in dpm mg^{-1} d.wt. and to the total P content in the mycorrhizal roots (Fig. 6a, b).

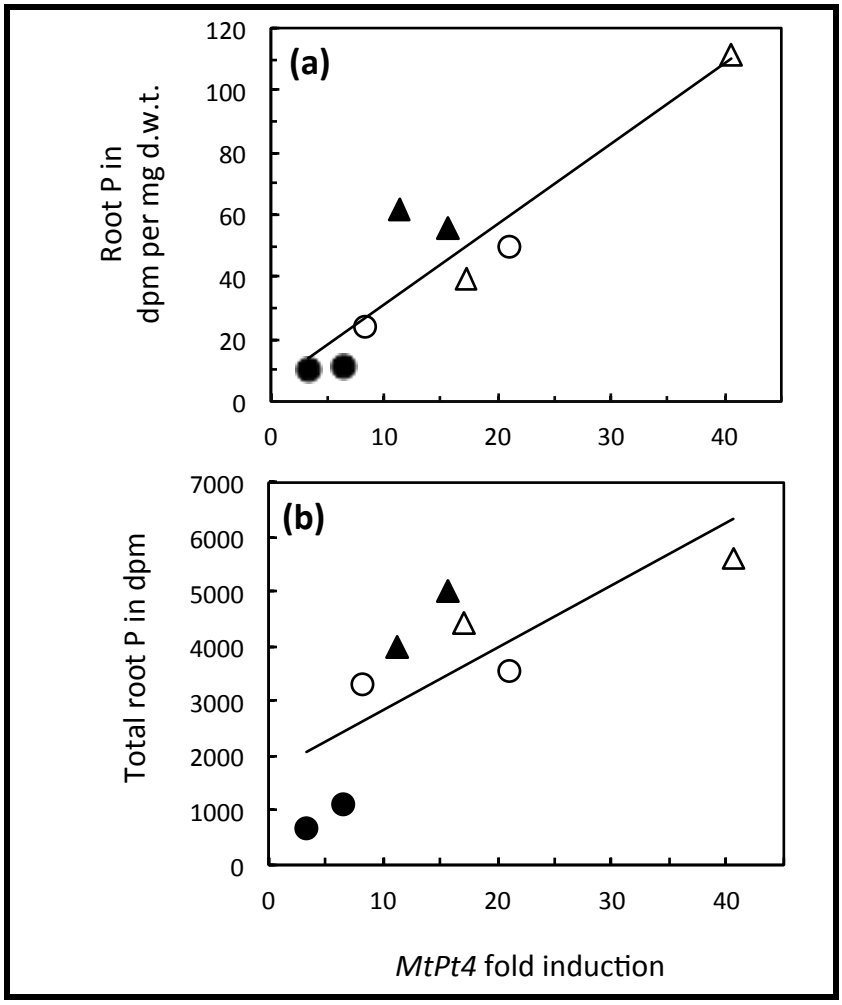

Fig. 6. Correlation between the *MtPt4* expression in mycorrhizal roots and the phosphate (P) transport to the roots in dpm per mg^{-1} d.wt. (a) and total dpm contents (b) of the roots. The *MtPt4* expression is shown in fold induction according to the $\Delta\Delta C_t$ method (Winer *et al.*, 1999). MtPt4 expression levels in roots colonized with *G. aggregatum* (triangles), or *R. irregularis* (circles) of non-shaded plants (open triangles or circles) or shaded plants (closed triangles or circles). Computed p-values of the correlation analysis for (a) $p = 0.0002$, $R^2 = 0.84535$; (b) $p = 0.0140$, $R^2 = 0.58762$.

1723.m00046, a putative ammonium transporter with a 99% sequence similarity to *Medicago truncatula* ammonium transporter 3 member MTR_8g074750 (NCBI XM_003629175.1) was induced in roots colonized with *G. aggregatum*, and the expression level was dependent on whether N or P was supplied to the CMN (Fig. 5c). When the CMN of *G. aggregatum* was supplied with N, *1723.m00046* was up-regulated up to 50-fold (compared to non-mycorrhizal roots), whereas when supplied with P, only a 2- to 3-fold induction was observed. We found no effect of the shading treatment on the transcript levels of *1723.m00046*. By contrast, the transcript levels of *1723.m00046* were comparatively low in roots that were colonized with *R. irregularis* and in shaded and non-shaded plants of NS/S systems lower than in non-mycorrhizal control plants (fold induction < 1) (Fig. 5d). We only found a 5- to 6-fold induction of *1723.m00046* in systems, in which two shaded (S/S) or non-shaded plants (NS/NS) shared one CMN that was supplied with N.

4.5 DISCUSSION

Underground, plants compete with other plants for nutrients provided by fungal CMNs, but the mechanisms that control the allocation patterns among plants are only poorly understood. We examined how nutrients supplied to the CMN were allocated between two host plants that differed in their ability to provide carbon resources. Specifically we asked if fungal partners were able to discriminate among hosts interconnected by a CMN. We found that when fungi were given a choice, they consistently allocated a higher percentage of both P and N to non-shaded hosts (Fig. 2), and that host plants that were restricted in their photosynthetic capability (here by shading) became relatively weak competitors for nutrient resources that were available from the CMN.

We varied the carbon source strength of the host plants by reducing the photosynthetically active radiation of half of the plants via shading. In systems in which the fungus had only access to shaded host plants (S/S), the mycorrhizal colonization was significantly reduced (N supply experiment; Fig. S3). This suggests that the shading treatment reduced the carbon supply for the CMN and that the AM fungus was unable to maintain its high colonization rate. *Medicago* has been shown to respond very sensitively to shading, and even a short-term shading can reduce the carbon allocation to the root system, and lead to a higher carbon allocation particularly to the shoot meristems to compensate for the decrease in the photosynthetic activity (Schmitt *et al.*, 2013). Shading for one to two weeks has been shown to reduce the mycorrhizal colonization of plants, but not to lower the carbon costs per nutrient benefit for the host plant (Heinemeyer *et al.*, 2004; Olsson *et al.*, 2010).

Interestingly, we found that shading did not reduce the mycorrhizal colonization rate of shaded plants when these plants shared a CMN with non-shaded host plants (NS/S). This suggests that the fungus used parts of the carbon derived from non-shaded host plants (or from its own reservoir in storage lipids) to maintain a high colonization rate in low quality hosts. This strategy potentially allows the obligate biotrophic fungus to maintain access to additional carbon sources, ensuring that the loss of a high quality host (e.g. by pathogen or herbivore damage) would be less detrimental. In the P supply experiment, the shading treatment was much shorter (six days in total), but still the resource allocation patterns indicated that both fungi were discriminating among the hosts. These data suggest that even before a significant reduction in the mycorrhizal colonization is expected (the whole arbuscular life cycle lasts around 8 d, the functionality for 2-3 d; Kobae & Hata, 2010), the fungus is able to change its nutrient allocation strategy in response to the shading treatment.

Previous studies using root organ cultures have shown that the carbon supply of the host acts an important trigger for P and N transport in the AM symbiosis (Bücking & Shachar-Hill, 2005; Hammer *et al.*, 2011; Kiers *et al.*, 2011; Fellbaum *et al.*, 2012). However, these systems have often been criticized for their artificial nature, most notably because the lack of a shoot in these systems prevents shoot-associated effects on nutrient uptake and sink strength (Smith & Smith, 2011). Another important difference is that in nature, CMNs can connect host plants of different ages and of multiple species (van der Heijden & Horton, 2009; Walder *et al.*, 2012). When large plants were grown with small seedlings, several studies have shown that the inter-connectedness to a large plant by a CMN can have a positive, negative or neutral effect on the growth of a smaller seedling

(van der Heijden & Horton, 2009). However, consistent to our results, Pietikäinen and Kytöviita (2007) reported that the mycorrhizal benefit for the seedling was low when the seedling shared a CMN with a non-defoliated adult plant (i.e. high quality host), but the benefit to the seedling began to increase when the carbon source strength of the adult plant was reduced by defoliation.

One could argue that the observed reduction in nutrient transport to shaded plants was the result of a lower plant nutrient demand. Low photosynthetic rates and the subsequent reduction in plant growth is expected to decrease the nutrient demand of the host (Cui & Caldwell, 1997). This is, however, unlikely in our experiment because the plants were grown under both P and N limitation before the shading treatment was started, and we found a preferential allocation of P to non-shaded host plants already after 6 days, when substantial differences in the growth between shaded and non-shaded plants were not expected. Even after shading for 24 days (N supply experiment), we found no significant difference in the plant biomass (Fig. S2), suggesting that differences in nutrient demand of the host did not play a large role in the observed nutrient allocation pattern.

We also analysed the expression of the AM-inducible P transporter *MtPt4* in the roots, and found that both fungi induced *MtPt4*, but that the induction by *G. aggregatum* was stronger than by *R. irregularis*. *MtPt4* is localized in the periarbuscular membrane (Pumplin *et al.*, 2012), and is involved in the P uptake from the mycorrhizal interface. *MtPt4* has been shown to be essential for the AM symbiosis, and in mutants in which this transporter was not expressed, arbuscules were prematurely degraded (Javot *et al.*, 2007). Mycorrhiza-inducible P transporters have been identified in several plant species, and high P availabilities for the host have been shown to reduce the transcript levels (Xu *et*

al., 2007; Breullin *et al.*, 2010). We found that the transcript levels of *MtPt4* were positively correlated to the P contents in mycorrhizal roots, and also indicated a higher P transport activity across the mycorrhizal interface. While a correlation also between *MtPt4* expression and arbuscular colonization cannot be completely excluded, the consistent colonization but differential transcript levels of *MtPt4* in shaded and non-shaded plants in NS/S systems, suggests that there is a direct correlation between *MtPt4* expression and transport activity. That a higher P transport to the root can also be coupled to an up-regulation of *MtPt4* was also shown by Fiorilli *et al.* (2013); the authors suggested that the P flux to the mycorrhizal host requires high expression levels.

The fact that shading reduced the expression of *MtPt4* is consistent with the predicted lower C allocation to the roots, and a reduction in the P transport across the mycorrhizal interface to low quality hosts. This supports the hypothesis that P and C transport across the mycorrhizal interface are tightly linked (Bücking & Shachar-Hill, 2005; Kiers *et al.*, 2011) and is consistent with the finding of Helber et al. (2011) that the expression of the fungal monosaccharide transporter *GintMST2*, suggested to be involved in the carbon uptake from the mycorrhizal interface, was positively correlated to the expression of *MtPt4*.

In contrast to the expression of *MtPt4*, shading did not result in a reduced expression of *1723.m00046*m. This is consistent with the relatively high transport of N to shaded plants we observed. However, our results demonstrate that both fungi can transfer substantial amounts of N to the host and that 23 days after $^{15}NH_4Cl$ was supplied to the CMN, a significant proportion of the N in the plants shoots was labeled. Due to the high mobility of N in the soil, the significance of the AM symbiosis for the N nutrition of the plant is

still under debate (for review see Smith & Smith, 2011). The work here, and of others (Toussaint *et al.*, 2004; Tanaka & Yano, 2005) demonstrates that AM fungi can contribute substantially to the N nutrition of plants. It is thought that the fungus transfers N in form of ammonium across the mycorrhizal interface to the host (Tian *et al.*, 2010; Fellbaum *et al.*, 2012). We found here that fungal N transport was coupled to an induction of *1723.m00046,* a putative ammonium transporter of *M. truncatula*. *1723.m00046* was first described by Gomez *et al.* (2009) and has been shown to be induced in the cortical cells of roots that were colonized with *R. irregularis*. We found that the transcript levels of *1723.m00046* in roots that were colonized with *R. irregularis* were lower than in roots that were colonized with *G. aggregatum*, but the 4- to 6-fold induction level in some of the *R. irregularis* treatments was consistent with the up-regulation observed by Gomez *et al.* (2009).

Our finding that this transporter was particularly up-regulated in roots that were associated with a CMN supplied with NH_4^+, supports the view that this transporter is potentially involved in the N uptake from the mycorrhizal interface. AM-inducible ammonium transporters that are localized in the periarbuscular membrane have been identified in several plant species (Kobae *et al.*, 2010; Koegel *et al.*, 2013). The AM-inducible ammonium transporter of *Lotus japonicus LjAMT2;2*, has been shown to transport NH_3 instead of NH_4^+, and it has been suggested that the protons from the NH_4^+ deprotonation remain in the interfacial apoplast and contribute to the H^+ gradient that facilitates proton-dependent transport processes across the mycorrhizal interface (Guether *et al.*, 2009).

Fungi can only preferentially allocate resources when there is a choice of high- vs. low quality plant hosts. We found that in *G. aggregatum* colonized systems, shaded plants connected with other shaded plants (S/S) received more resources from the CMN than shaded plants that had to compete with non-shaded host plants (NS/S). Following biological market dynamics (Kiers *et al.*, 2011), this finding suggests that in the absence of choice, *G. aggregatum* transfers more resources per unit carbon to low quality hosts, and that the higher carbon demand of the fungus shifts the cost to benefit ratio in favor of the host (under the assumption that carbon transport of shaded plants to the CMN did not differ between S/S or NS/S systems). Since the mycorrhizal colonization was reduced in systems with two shaded plants (S/S), this indicates that the P and N transport rate *per unit interface* increased under these conditions. This supports the findings of Treseder (2013), who reported that mycorrhizal growth responses not only depend on the mycorrhizal colonization, but also on the mycorrhizal benefits provided per unit root length colonized.

Our findings support the hypothesis that the fungus is more in control than previously thought, despite its obligate dependence on the host. Often, the plant host is considered to be more in control of mycorrhizal outcomes. This is because, in contrast to the AM fungus, many plant species are not obligately dependent on the symbiosis (Smith & Smith, 2012), and reduce their mycorrhizal colonization rate actively by a premature degeneration of arbuscules. This has particularly been demonstrated in cases when the P availability was high, or when the plant was unable to benefit from the P transport across the mycorrhizal interface (Javot *et al.*, 2007; Breullin *et al.*, 2010). It has been suggested, on the other hand, that the fungus can actively control the transport of P and N into the

mycorrhizal interface by the regulation of poly-P formation and/or remobilization in the IRM (Bücking & Shachar-Hill, 2005; Ohtomo & Saito, 2005; Takanishi *et al.*, 2009). This is consistent with our finding that in the roots of shaded plant hosts colonized by *R. irregularis* (but not *G. aggregatum*), a significantly higher proportion of the total poly-P was stored in the form of long-chained poly-P (Fig. S4). Long-chained poly-P better represent the long-term storage capacity of P in AM fungal hyphae, whereas short-chained poly-P, are seen as a good indicator for P transport to the host (Takanishi *et al.*, 2009; Kiers *et al.*, 2011). The fungus could also potentially control its nutrient transport to the host via a differential expression of transporters in the arbuscular membrane. The expression of fungal phosphate and ammonium transporters in the arbuscular membrane suggests that both partners, plant and fungus, compete for P and N that becomes available in the interfacial apoplast (Balestrini *et al.*, 2007; Pérez-Tienda *et al.*, 2011).

While we found strong evidence that both fungal partners successfully discriminated among hosts of different quality, both fungi still transferred substantial amounts (~20 - 40 %) of P and N to low quality hosts. Detailed studies on the arbuscular lifespan in roots are limited, but fungal arbuscules undergo in host cells a cycle of growth, maturity, senescence and recurrent growth; it has been suggested that the turnover of arbuscules potentially provides the host plant with an instrument ‘to penalize’ inefficient fungal symbionts (Javot *et al.*, 2007; Parniske, 2008). A low but continuous flux of nutrients to low quality hosts would allow the fungus to escape arbuscular degradation. This, in turn, decreases the dependency of the fungus on a specific host. Multiple host plants that contribute to the C supply and compete for limited resources available for the CMN will

likely shift the cost to benefit ratio in favor of the fungus, as increasing the number of hosts would give the fungus more bargaining power.

4.6 CONCLUSION

AM associations are a perfect illustration of mutualisms involving many-to-many interactions: plants are typically colonized by AM fungal communities of multiple species, and fungal "individuals" form a CMN and simultaneously colonize multiple host plants and species. Understanding the trading and distribution of resources is a key question for the AM symbiosis, and mutualisms in general. We examined here how plants compete for limited resources that become available for the CMN, and how fungal symbionts regulate the nutrient allocation to multiple host plants. Our current understanding of resource exchange and cost to benefit relationships in the AM symbiosis is mainly based on experiments with *in vitro* root organ cultures or studies that were performed with single plants lacking mycelial inter-connections to other plants. These only poorly represent nutrient and resource allocation under natural conditions when multiple plants compete for resources from the CMN (van der Heijden & Horton, 2009). We demonstrate here in a whole plant system that both fungi preferentially allocated nutrient resources to host plants that were able to provide more benefit. This is consistent with previous reports from *in vitro* root organ cultures, in which the carbon supply of the host was shown to act as an important trigger that stimulates fungal P and N transport (Bücking & Shachar-Hill, 2005; Hammer *et al.*, 2011; Kiers *et al.*, 2011; Fellbaum *et al.*, 2012; Fellbaum *et al.*, 2012).

The plant has often been considered to be more in control of the mycorrhizal outcomes than the fungal symbiont. Our results suggest that the fungal partner, although an obligate biotroph, still retains power via its ability to change nutrient allocation patterns. However, we also found that in the absence of choice, fungi (e.g. *G. aggregatum*) transfer more resources per unit carbon to low quality hosts, shifting the cost to benefit ratio in favor of the host. Our studies also support the hypothesis that carbon to nutrient exchange ratios at the mycorrhizal interface follow biological market dynamics, that depend on the compatibility between the plant and fungal species involved (Smith *et al.*, 2004), and resource supply and demand conditions (Kiers *et al.*, 2011; Fellbaum *et al.*, 2012). Because we demonstrated the importance of both N and P allocation patterns, future studies should track both resources simultaneously to understand the market dynamics of multiple nutrient commodities and how costs and benefits of the symbiosis (Johnson *et al.*, 1997; Johnson & Graham, 2013) manifest within complex CMNs.

4.7 ACKNOWLEDGEMENTS

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4.9 SUPPLEMENTARY INFORMATION

4.9.1 FIGURES

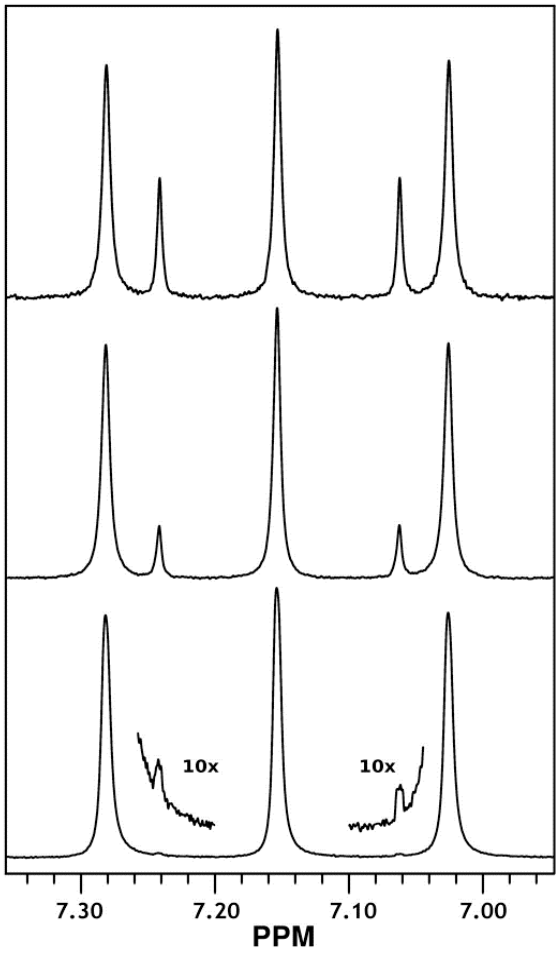

Fig.S1. 400MHz ^{1}H NMR spectra of Kjeldahl degraded tissue of non-mycorrhizal roots (a), mycorrhizal roots of shaded plants (b), or mycorrhizal roots of non-shaded plants (c).

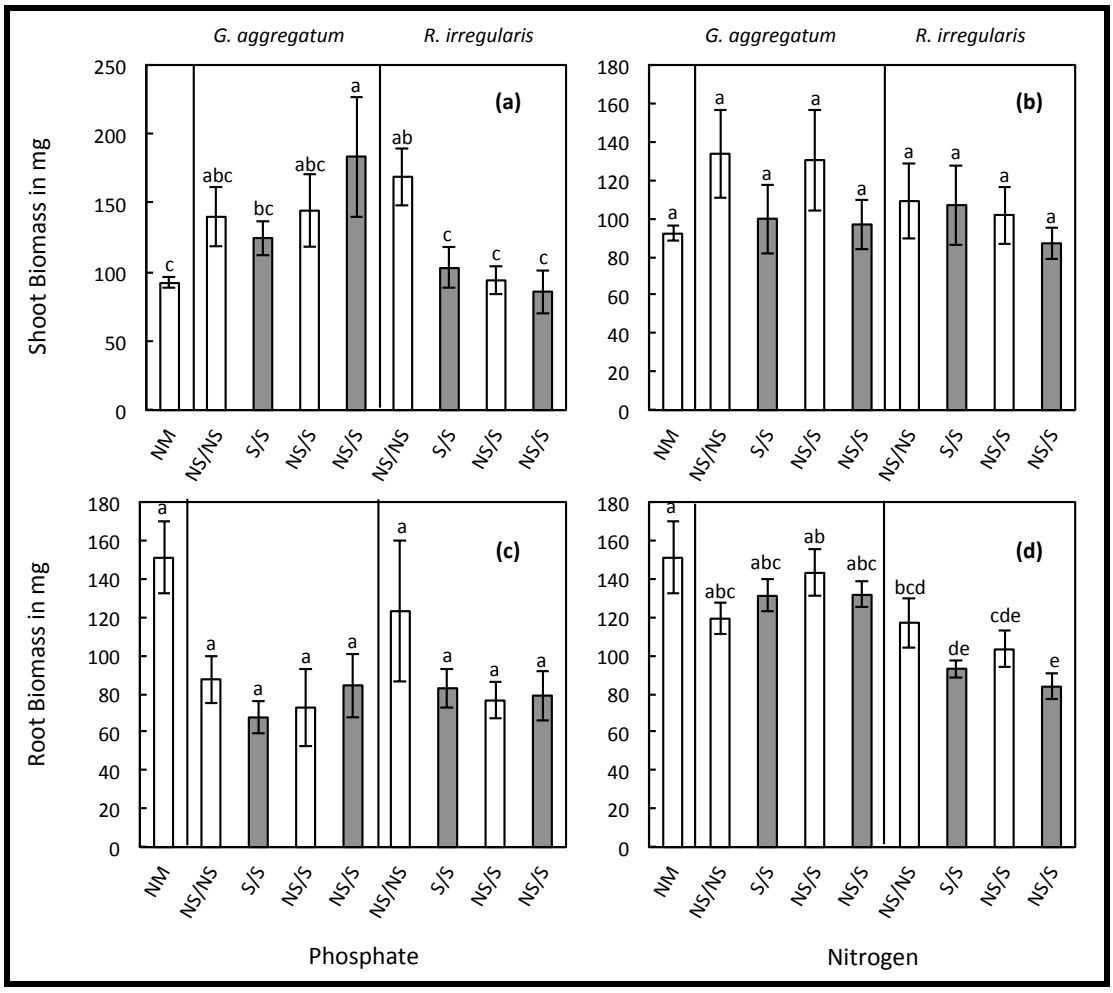

Fig. S2. Effect of mycorrhizal colonization and shading treatment on shoot (a, b) and root (c, d) biomass of shaded (grey bars). Non-shaded (white bars) mycorrhizal and non-mycorrhizal (NM) plants of the ^{33}P labeling experiment (a,c) and ^{15}N labeling experiment (b,d). It should be noted that NM control plants were not clearly comparable to the mycorrhizal treatments, because control plants did not have access to the nutrients that were supplied to the FC. Different letters on the bars indicates statistically significant differences according to the one-way ANOVA and LSD test ($P \leq 0.05$). The results of the one-way ANOVA are showed in Table S5.

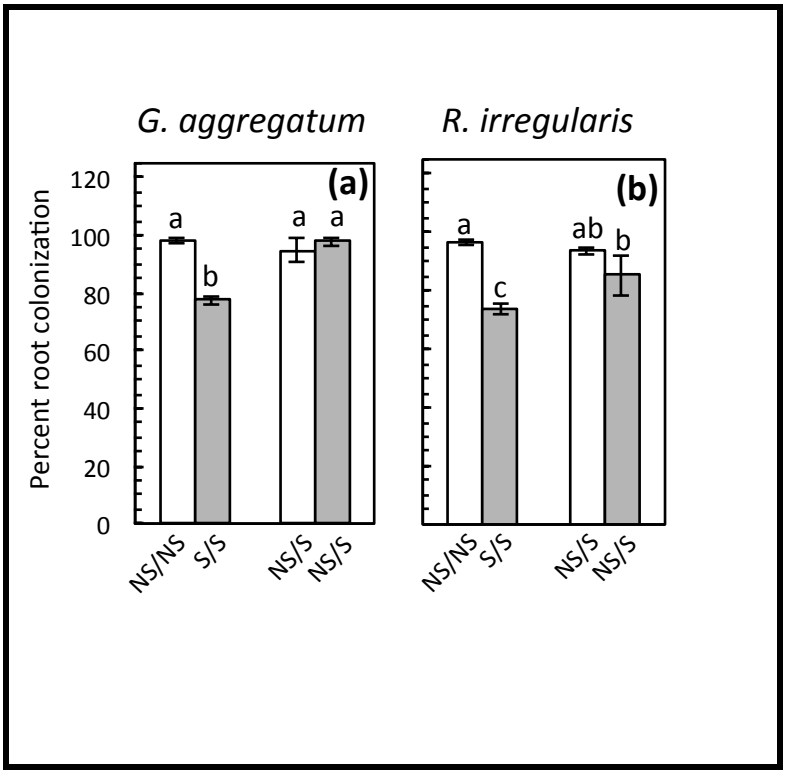

Fig. S3. Mycorrhizal colonization with *G. aggregatum* (a) or R. irregularis (b) of non-shaded (white bars) or shaded (grey bars) plants that were grown in systems with two non-shaded plants (NS/NS), two shaded plants (S/S) or one non-shaded and one shaded plant (NS/S). Shown is the average of n = 5-10 ± SE. Different letters on the bars indicates statistically significant differences according to the one-way ANOVA and LSD test ($P \leq 0.05$). The results of the one-way ANOVA are showed in Table S6.

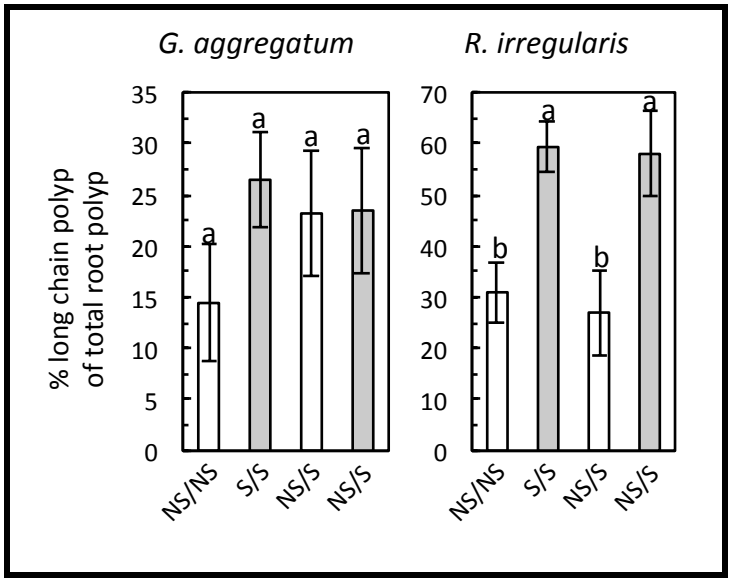

Fig. S4. Percentage of long-chained poly-P of the total poly-P in mycorrhizal roots colonized with *G. aggregatum* (a) or *R. irregularis* (b) in non-shaded (white bars) or shaded (grey bars) plants that were grown in systems with two non-shaded plants (NS/NS), two shaded (S/S) or one non-shaded and one shaded plant (NS/S). Shown is the average of n = 5-10 ± SE. Different letters on the bars indicates statistically significant differences according to the one-way ANOVA and LSD test ($P \leq 0.05$). The results of the one-way ANOVA are showed in Table S7.

4.9.2 TABLES

Table S1. Results of two-way ANOVA: Effect of shade treatment or fungal species on ERM development in the fungal compartment of the growth systems.

		F	*P*
Fungal species	$F_{1,11}$=	2.8578	0.119
Shade treatment	$F_{1,11}$=	0.0364	0.8521
Fungal species x Shade treatment	$F_{1,11}$=	0.0001	0.9921

Table S2. Results of two-way ANOVA: Effect of shade treatment or fungal species on ^{33}P contents in dpm mg^{-1} d.wt. in roots and shoots of plants interconnected by a CMN (see also Fig. 3).

Root:

Factor		F	*P*
Fungal species	$F_{1,50}$=	10.3113	0.0023
Shade treatment	$F_{3,50}$=	2.6421	0.0594
Fungal species x Shade treatment	$F_{3,50}$=	0.6032	0.616

Shoot:

Factor		F	*P*
Fungal species	$F_{1,50}$=	0.0149	0.9033
Shade treatment	$F_{3,50}$=	5.8961	0.0016
Fungal species x Shade treatment	$F_{3,50}$=	1.8986	0.1418

Table S3. Results of two-way ANOVA: Effect of shade treatment or fungal species on ^{15}N contents in % of total N in roots and shoots of plants interconnected by a CMN (see also Fig. 4).

Root:

Factor		F	*P*
Fungal species	$F_{1,32}$=	4.235	0.0478
Shade treatment	$F_{3,32}$=	5.7783	0.0028
Fungal species x Shade treatment	$F_{3,32}$=	3.8623	0.0183

Shoot:

Factor		F	*P*
Fungal species	$F_{1,32}$=	2.4332	0.1286
Shade treatment	$F_{3,32}$=	3.1936	0.0366
Fungal species x Shade treatment	$F_{3,32}$=	1.8809	0.1527

Table S4. Results of three-way ANOVA: Effect of shade treatment, fungal species, or nutrient addition to the FC on the expression of *MtPt4* or *1723.m00046* in the roots of *Medicago truncatula* (see also Fig. 5).

MtPt4		F	*P*
Fungal species	$F_{1,76}$=	29.0298	<.0001
Shade treatment	$F_{3,76}$=	10.7434	<.0001
Fungal species x Shade treatment	$F_{3,76}$=	4.5835	0.0053
Nutrient supply	$F_{1,76}$=	15.6099	0.0002
Fungal species x Nutrient supply	$F_{1,76}$=	7.528	0.0076
Shade treatment x Nutrient supply	$F_{3,76}$=	2.3015	0.0839
Fungal species x Shade treatment x Nutrient supply	$F_{3,76}$=	2.1213	0.1045

1723.m00046		F	*P*
Fungal species	$F_{1,77}$=	23.57	<.0001
Shade treatment	$F_{3,77}$=	2.5175	0.0643
Fungal species x Shade treatment	$F_{3,77}$=	5.5765	0.0016
Nutrient supply	$F_{1,77}$=	28.7949	<.0001
Fungal species x Nutrient supply	$F_{1,77}$=	18.2394	<.0001
Shade treatment x Nutrient supply	$F_{3,77}$=	2.9223	0.0392
Fungal species x Shade treatment x Nutrient supply	$F_{3,77}$=	3.9984	0.0106

Table S5. Results of one-way ANOVA: Biomass of root and shoots of non-mycorrhizal and mycorrhizal plants of the ^{15}N or ^{33}P labeling experiment (see also Fig. S2).

		F	*P*
Roots, ^{15}N			
Treatment	$F_{8,55}$=	3.9099	0.001
Roots, ^{33}P			
Treatment	$F_{8,50}$=	1.4427	0.2024
Shoot, ^{15}N			
Treatment	$F_{8,59}$=	0.629	0.7501
Shoots, ^{33}P			
Treatment	$F_{8,54}$=	2.9104	0.009

Table S6. Results of two-way ANOVA: Effect of shade treatment and fungal species on mycorrhizal root colonization of *Medicago truncatula* (see also Fig. S3).

		F	*P*
Fungal species	$F_{1,36}$=	5.4253	0.0256
Shade treatment	$F_{3,36}$=	27.3573	<.0001
Fungal species x Shade treatment	$F_{3,36}$=	1.4638	0.2407

Table S7. Results of two-way ANOVA: Effect of shade treatment and fungal species on the percentage of long-chained polyP of the total root polyp in the *Medicago truncatula* (see also Fig. S4).

		F	*P*
Fungal species	$F_{1,45}$=	23.1404	<.0001
Shade treatment	$F_{3,45}$=	27.3573	0.0012
Fungal species x Shade treatment	$F_{3,45}$=	2.424	0.0781

CHAPTER 5: HIGH FUNCTIONAL DIVERSITY WITHIN SPECIES OF ARBUSCULAR MYCORRHIZAL FUNGI IS ASSOCIATED WITH DIFFERENCES IN PHOSPHATE AND NITROGEN UPTAKE AND FUNGAL PHOSPHATE METABOLISM

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*I contributed to P & N nutrition analysis, manuscript writing and revisions

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5.1 ABSTRACT

Plant growth responses following colonization with different isolates of a single species of an arbuscular mycorrhizal (AM) fungus can range from highly beneficial to detrimental, but the reasons for this high within-species diversity are currently unknown. To examine whether differences in growth and nutritional benefits are related to the phosphate (P) metabolism of the fungal symbiont, the effect of 31 different isolates from 10 AM fungal morphospecies on the P and N nutrition of *Medicago sativa* and the P allocation among different P pools was examined. Based on differences in the mycorrhizal growth response, high, medium and low performance isolates were distinguished. Plant growth benefit was positively correlated to the mycorrhizal effect on P and N nutrition. High performance isolates increased plant biomass by more than 170 %, and contributed substantially to both P and N nutrition, whereas the effect of medium performance isolates particularly on the N nutrition of the host was significantly lower. Roots colonized by high performance isolates were characterized by relatively low tissue concentrations of inorganic P and short-chain polyphosphates, and a high ratio between long- to short-chain polyphosphates. The high performance isolates belonged to different morphospecies and genera, indicating that the ability to contribute to P and N nutrition is widespread within the Glomeromycota, and that differences in symbiotic performance and P metabolism are not specific for individual fungal morphospecies.

5.2 INTRODUCTION

Arbuscular mycorrhizal (AM) fungi form mutualistic interactions with approximately 65% of all known land plant species (Wang & Qiu 2006), and are among the most ecologically important soil microbes in natural and agricultural ecosystems. The extraradical mycelium (ERM) of the fungus acts as an extension of the root system and takes up phosphate (P), nitrogen (N), sulfur and trace elements from the soil, and delivers these nutrients via the intraradical mycelium (IRM) to the plant (Smith & Smith 2011; Allen & Shachar-Hill 2009; Hawkins *et al.,* 2000; Jakobsen *et al.,* 1992). In exchange, the plant allocates up to 20 % of its photosynthetically fixed carbon to the fungus (Wright *et al.,* 1998). This carbon supply acts as an important trigger for P and N transport in the AM symbiosis (Fellbaum *et al.,* 2012b; Bücking & Shachar-Hill 2005; Hammer *et al.,* 2011; Fellbaum *et al.,* 2014), and it has been demonstrated that both host and fungus can discriminate among their partners, reciprocally rewarding those partners that provide more mutualistic benefit (Kiers *et al.,* 2011).

While the symbiosis is generally positive for the host, mycorrhizal growth responses (MGR) can range from highly beneficial to detrimental (Johnson & Graham 2013; Johnson *et al.,* 1997; Smith & Smith 2013) depending on abiotic factors such as nutrient level (Smith & Smith 2013; Peng *et al.,* 1993; Nouri *et al.,* 2014), and biotic factors such as the identity of the fungal symbiont colonizing the host (Smith *et al.,* 2004). There is a high functional diversity in nutritional benefit, not only among different fungal morphospecies, but also among isolates within one morphospecies, and it has been shown that even the genetic diversity in one initial spore can be sufficient for the

development of phenotypically different variants of one fungus (Ehinger *et al.,* 2012). While fungal isolates differ greatly in the efficiency with which they provide nutritional benefits to plant hosts (Avio *et al.,* 2009; Avio *et al.,* 2006; Hart & Reader 2002b), there still lacks a clear understanding why particular AM fungal isolates are much more beneficial than others.

When inorganic phosphate (P_i) is taken up by the ERM, it can first replenish the metabolically active P_i pool in the hyphae that will for example be used for the synthesis of phospholipids, DNA-, RNA- or protein-phosphates, or it can be converted into long-chained or short-chain polyphosphates (poly-P). Poly-P are linear polymers in which up to several hundred P_i residues are linked by energy-rich phospho-anhydride bonds. Poly-P are rapidly synthesized in the hyphae of the ERM (Ezawa *et al.,* 2003) presumably by the poly-P polymerase/vacuolar transporter chaperone complex (VTC; Tisserant *et al.,* 2012), and this poly-P accumulation is followed by a near-equivalent cation uptake by the fungal hyphae (Kikuchi *et al.,* 2014). Poly-P play an important role in the storage of P in the fungal hyphae, but also in the translocation of P from the ERM to the IRM (Hijikata *et al.,* 2010). In the IRM long-chain poly-P are broken down first into shorter chain lengths by a vacuolar endopolyphosphatase, followed by an exopolyphosphatase that hydrolyzes the terminal residues from the short-chain poly-P and releases P_i that can be transferred across the mycorrhizal interface to the host (Tisserant *et al.,* 2012; Ezawa *et al.,* 2001).

Inorganic N sources taken up by the fungus from the soil are assimilated in the hyphae of the ERM and converted mainly into the basic amino acid arginine (Cruz *et al.,* 2007; Jin *et al.,* 2005). It has been suggested that arginine could bind to the negatively

charged poly-P and could be transferred with poly-P from the ERM to the IRM (Cruz *et al.,* 2007; Fellbaum *et al.,* 2012a). In the IRM, poly-P are remobilized and P_i and arginine are released, and the catabolic arm of the urea cycle re-converts arginine back into NH_4^+ (Govindarajulu *et al.,* 2005; Fellbaum *et al.,* 2012a; Tian *et al.,* 2010). P_i and NH_4^+ are then transferred into the mycorrhizal interface and are taken up from the interface by mycorrhiza-inducible plant P and ammonium transporters that are localized in the periarbuscular membrane (Gomez *et al.,* 2009; Guether *et al.,* 2009; Javot *et al.,* 2007; Pumplin *et al.,* 2012).

Considering the important role that poly-P play in P and N transport in the AM symbiosis, more knowledge about the poly-P metabolism and remobilization may contribute to a better understanding of the differences in the growth and nutritional benefits conferred by diverse fungal isolates. AM fungi differ in their poly-P metabolism (Boddington & Dodd 1999) and the regulation of poly-P formation and/or remobilization in the IRM provides the fungus with an instrument to regulate the P and N transport into the mycorrhizal interface (Bücking & Shachar-Hill 2005; Ohtomo & Saito 2005; Takanishi *et al.,* 2009). To test this idea, we have studied the P and N nutrition and the P pool distribution in *Medicago sativa* after colonization with 31 different AM fungal isolates, and determined whether nutritional benefits to the host were correlated to the P metabolism of the fungus. Use of this diverse fungal collection allowed comparison of intra- and interspecific functional variability in the P metabolism of AM fungi across the phylum Glomeromycota, and insight into whether differences in fungal P metabolism are related to the fungal phylogeny and whether these differences affect the nutritional benefits for the host.

5.3 MATERIAL AND METHODS

Fungal and Plant culture

Medicago sativa L. (alfalfa) was selected as a host plant because this species is highly dependent on mycorrhizal interactions, and it shows high functional compatibility with AM fungal symbionts (Monzon & Azcon 1996; Chen *et al.,* 2007). The plants were inoculated with 31 different AM fungal isolates from 6 different families, 7 genera, and 10 AM fungal morphospecies. The majority of the fungal isolates were obtained from the International Culture Collection of Arbuscular Mycorrhizal Fungi (INVAM; http://invam.wvu.edu), except *Rhizophagus irregulare* (previously *Glomus intraradices*) that was isolated from root organ cultures (Koch *et al.,* 2004). Some AM fungal taxa were recently phylogenetically re-classified and re-named based on SSU rRNA sequencing (Schüßler & Walker 2010). Since the AM fungal classification is still under debate, and the exact species affiliation of the *Rhizophagus intraradices* isolates is uncertain, *R. intraradices* and *R. irregulare* (R. *irregulare* corresponds to *Glomus intraradices* DAOM197198, Stockinger *et al.,* 2009) was considered as one species. Table 1 includes the fungal morphospecies and isolates with their old and new species affiliation.

The alfalfa seeds were surface sterilized for 1 min in 7 % bleach, and rinsed three times with sterile water, before sowing. Plants were grown in pots filled at the bottom with 50 ml autoclaved (twice at 121°C for 20 min) and pressed Sunshine mix #2 (Sun Gro Horticulture, Vancouver, BC, Canada), which was overlayed with 100 ml of an autoclaved (see above) mixture (1:3:1; v:v:v) of field soil, Turface (Turface Athletics

MVP, Profile Products LLC, Buffalo Grove, IL, USA) and washed horticultural sand (Hillview, Nu-Gro IP Inc., Brantford, ON, Canada), 20 ml of non-mycorrhizal or mycorrhizal inoculum (see below), and on top with another 50 ml of the substrate mixture. The field soil was collected at the Long-Term Mycorrhizal Research Site located at the University of Guelph (Canada) (Kliromonos 2000), passed through a 5 mm sieve and air-dried at room temperature. The chemical properties of the field soil (analyzed by the University of Guelph Laboratory Services, ON, Canada) were: 140 mM kg-^{1} total N (measured by LECO FP 428 N analyzer), 0.065 mM kg^{-1} available P (Olsen method), pH 7.7 (saturated paste method).

The mycorrhizal inoculum for the experiment was produced by growing each fungal isolate with *Sorghum vulgare* (Pers.) var. *sudanense* as host species in pot cultures in a greenhouse at the University of Guelph (Canada). The substrates of these cultures were collected after 5 months, air-dried, and controlled for the presence of viable AM fungal spores of the correct morphotype. To each of the mycorrhizal treatments, 20 ml of inoculum containing AM fungal spores, hyphae and mycorrhizal roots were added. To the non-mycorrhizal controls, 20 ml of substrate and roots of non-mycorrhizal *S. vulgare* cultures or 20 ml of autoclaved fungal inoculum was added. No signs of AM fungal colonization (no root colonization, no fungal spores) were found in either control treatment, and since both control treatments did not differ statistically in any of the traits studied, they were subsequently pooled into one non-mycorrhizal control group. To minimize differences in the non-AM microbial communities, 1 ml of a microbial wash solution was added to each container. This microbial wash solution was obtained by suspending 20 ml subsamples of each of the AM fungal inocula from the *Sorghum*

vulgare cultures (see above) in 2 l of sterile water, and by filtering the solution through a 20 µm sieve. All containers were covered with a thin layer of sterile washed sand and arranged in a completely randomized block design in the greenhouse.

One week after seed germination, the seedlings were manually reduced to three and then to one single plant per pot after 3 weeks. The plants were watered every 2 to 3 days with de-ionized water, and fertilized with 10 mg of a low P fertilizer (17-5-19; Antunes *et al.,* 2011) after 8, 12 and 16 weeks (in total 5.1 mg total N, 1.5 mg P_2O_5, and 2.7 mg K_2O). The temperature in the greenhouse ranged between 16 to 18°C at night and 23 to 26°C during the day and artificial light was added when necessary. The plants were harvested after 20 weeks, before they became root-bound, to ensure that all fungal isolates, independent of their inoculation strength, had sufficient time to colonize the root system. At harvest, fungal and plant growth characteristics were determined, and the samples were prepared for N and P analysis.

Table 1. List of the AM fungal species and isolates used for the experiment (classification according to Schüßler and Walker 2010). Several of the fungal species have recently been re-classified and re-named and the former species name is given in brackets.

Order	Family	Genus	Species	Abbreviation and name of the isolate
Glomerales	Glomeraceae	*Rhizophagus (Glomus)*	*irregulare*	Rhi irr QB000
		Rhizophagus (Glomus)	*intraradices*	Rhi int ON.pr.Te3
				Rhi int KE103
				Rhi int TU101
		Funneliformis (Glomus)	*mosseae*	Fun mos HO102
				Fun mos CU114
				Fun mos NB114
	Claroideoglomeraceae	*Claroideoglomus (Glomus)*	*claroideum*	Cla cla UT159A
				Cla cla DN987
				Cla cla BR106
			etunicatum	Cla etu MX116A
				Cla etu MG106
				Cla etu SP108C
Diversisporales	Gigasporaceae	*Gigaspora*	*margarita*	Gig mar JA201A
				Gig mar MR104
				Gig mar WV205A
	Acaulosporaceae	*Acaulospora*	*scrobiculata*	Aca scr CU130
				Aca scr BR602
				Aca scr VA104
			morrowiae	Aca mor CR207
				Aca mor EY106
				Aca mor FL219B
		Acaulospora (Entrophospora)	*colombiana*	Aca col CL356
				Aca col GA101
				Aca col NB104C
Paraglomerales	Paraglomeraceae	*Paraglomus (Glomus)*	*occultum*	Par occ CR102
				Par occ HA771
				Par occ OR924
Archaeosporales	Ambisporaceae	*Ambispora (Glomus)*	*leptoticha*	Amb lep FL130A
				Amb lep JA401A
				Amb lep CR312

Analysis of fungal and plant growth characteristics

At harvest, root and shoot biomass of the plants was assessed, roots were examined for root nodules, and the dry weight of the nodulated root parts was determined. Fungal growth characteristics such as the percentage root length colonized by arbuscules, vesicles and hyphae (%AC, %VC and %HC, respectively), the number of AM fungal spores and the hyphal length per g substrate were examined using standard protocols (Klironomos *et al.,* 1993; McGonigle *et al.,* 1990; Miller *et al.,* 1995). The percentage mycorrhizal growth responses (MGR) in terms of total plant biomass were determined based on the dry weights (d.wt.) of individual mycorrhizal plants and the mean d.wt. of the non-mycorrhizal controls, using the following formula:

MGR in % = 100 x (d.wt. AM plant – d.wt. mean of controls) / d.wt. mean of controls.

Phosphate and Nitrogen analysis

Root and shoot samples were individually homogenized in a tissue grinder (Precellys 24, Cayman Chemical Compony, Ann Arbor, USA), and an aliquot of each sample was dried and analysed for P or N content. For the P analysis, the sample was extracted with 2 N HCl at 95°C for 1 h (Ohtomo *et al.,* 2004). Additionally, the allocation of P into different P pools in non-mycorrhizal and mycorrhizal root samples was measured following the protocol described by Aitchison and Butt (1973). The samples were dried at 70°C, weighed and analysed for the following P pools: P_i and acid soluble or short-chain poly-P (chain length ≤ 20 P_i residues) after extraction with ice-cold 10 % TCA, phospholipids after extraction with 100 % ethanol and ethanol/ether (3:1, v:v), acid-insoluble poly-P (chain length > 20 P_i residues) after extraction with 1 M KOH, and DNA-, RNA- and

protein-phosphates as residue after extraction of all other pools. The pH of the supernatants of the TCA or KOH extractions containing the acid soluble or acid insoluble poly-P were first neutralized by adding 3 M KOH or 3 M HCl, respectively, and then adjusted to a pH of 4.5 by adding 3 M acetate buffer. The poly-P were then precipitated twice by adding a saturated $BaCl_2$ solution at 4°C overnight. An aliquot of the poly-P or DNA-, RNA-, protein-phosphate precipitates was diluted in 2 N HCl and heated up to 95°C for 1 h before analysis. The P content was measured spectrophotometrically at 436 nm after adding ammonium-molybdate-vanadate solution (Ricca Chemical, Arlington, TX, USA) to an aliquot of the sample. The total N content in 3 mg aliquots of the shoots was analysed by using an isotope mass spectrometer (Sercon, Europa-Scientific, Crewe, UK).

Statistical Analysis

The data are based on four biological replicates per AM fungal isolate and eight non-mycorrhizal control plants. Since the results demonstrated high intraspecific variability and the species affiliation for several of the fungal species is uncertain at this point, all fungal isolates were treated as independent variables in all statistical tests. Unless mentioned otherwise, treatment effects are only discussed when they were statistically significant according to one-way ANOVA with isolate as a fixed factor followed by Fisher´s Least Significant Difference (LSD) test ($p \leq 0.05$) (biomass data). An ANCOVA was used to confirm the results of the ANOVA analysis and to account for the effects of the continuous co-variate (biomass) on the statistical evaluation of the nutritional

benefits. The results of these tests are given in the Tables S1 to S7 (see supplementary information).

The fungal isolates were grouped according to their effect on the plant MGR as high, medium, and low performance isolates (see results). The fungal isolates were grouped according to their performance and an additional one-way ANOVA and LSD test was conducted only when ANOVA and LSD test of the individual fungal isolates demonstrated significant differences between fungal isolates that were related to their symbiotic performance. Correlations among traits were examined by calculating the Pearson correlation coefficient or a linear regression analysis ($p \leq 0.05$). These results are shown in the Tables S8 to S11. The analytical software UNISTAT 6 (Unistat Ltd., London, U.K.) was used for all analyses.

5.4 RESULTS

Effect of different AM fungi on plant biomass

Total biomass of mycorrhizal *M. sativa* plants was higher than that of non-mycorrhizal control plants, but there was a high variability in the mycorrhizal growth response (MGR) across AM fungal isolates (Fig. 1, Table S1). The % increase in total plant biomass ranged from 7.3 ± 10.8 (mean ± S.E.M.) in plants colonized with *Rhizophagus irregulare* QB000 (not significantly higher than the controls) to 207.4 ± 36.4 in plants colonized with *Acaulospora colombiana* NB104C. The intraspecific variability in the MGR between the different isolates of one AM fungal morphospecies was similarly high. For example, two other strains of *A. colombiana* (CL 356 and GA101) did not lead to a significant growth response relative to the non-mycorrhizal controls. Of the fungal

isolates tested, the three *A. morrowiae* isolates led on average to the highest increase (113.8 ± 32.4 %) and the four *Rhizophagus* isolates to the lowest increase in total plant biomass (20.2 ± 15.9 %) (Table S1).

Among the different fungal isolates that were tested, six stood out because they showed several unique characteristics (in e.g. their effect on P and N nutrition), and resulted in the highest increase in total plant biomass relative to all other isolates (i.e., a MGR of more than 170 % relative to the control, and more than 65 % higher than the next highest performing isolate with an increase in host biomass of 104 %) (Fig. 1, Table S1, Table S8[1-7]). This group, later referred to as "high performance isolates", included isolates from six different fungal morphospecies, *A. colombiana* NB104C, *Funneliformis mosseae* NB114, *A. morrowiae* FL219B, *Paraglomus occultum* OR924, *A. scrobiculata* VA104, and *Claroideoglomus etunicatum* MG106. Six isolates led only to small increases in total plant biomass (≤ 18 %) and did not differ significantly in many characteristics from the non-mycorrhizal controls but differed from the high performance isolates. These "low performance isolates" included *Rhizophagus irregulare* QB000, *P. occultum* CR102, *C. claroideum* UT159A, *A. scrobiculata* CU 130, *R. intraradices* ON.pr.Te3, and *A. colombiana* CL356 (Table S1). In between the low and high performance isolates, a group of isolates could be identified that significantly increased plant biomass compared to the controls, but led to a significantly lower biomass response than the high performance isolates (Fig. 1, Table S1). These "medium performance" isolates led to MGR between 71.7 % and 104.0 % and included *Ambispora leptoticha* CR312, *P. occultum* HA771, *Gigaspora margarita* WV205A, *C. etunicatum* SP108C, *A. morrowiae* EY106, *C. claroideum* BR106, and *A. morrowiae* CR207. A high within

treatment variability in plant growth responses was observed for the remaining isolates. Plants colonized by these isolates did not differ significantly from the non-mycorrhizal controls, but had a consistently lower biomass response than the high performance isolates.

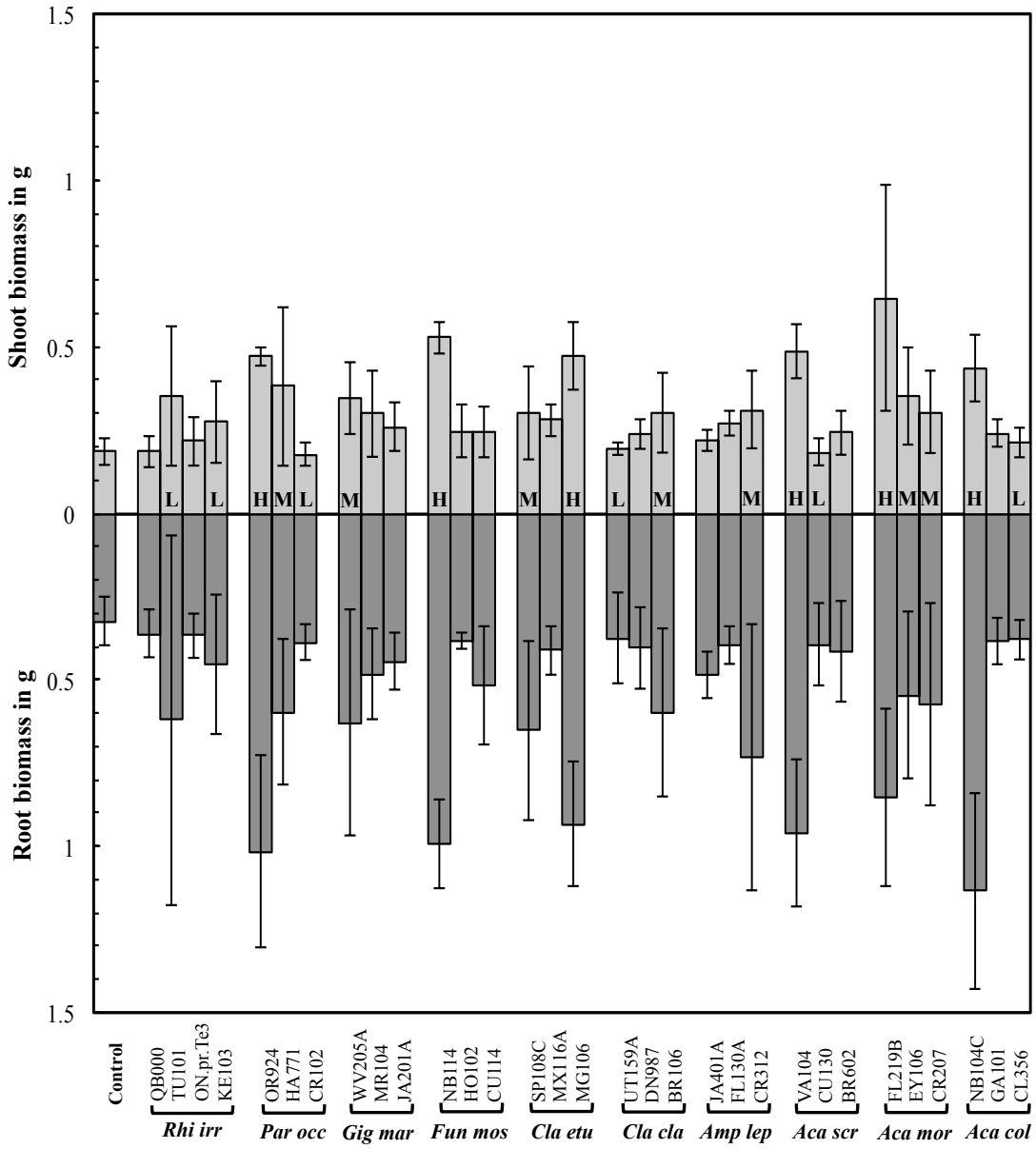

Fig. 1. Plant biomass characteristics of non-mycorrhizal and mycorrhizal *Medicago sativa* plants. The bars show the means (n=4) of the dry biomass in g of roots (bottom, dark grey) and shoots (top, light grey) and their respective confidence intervals ($p \leq 0.05$). The letters in the bars indicate whether the isolate belonged to the low (L), medium (M), or high (H) performance isolates.

Correlation between host biomass and P benefits of the AM symbiosis

Mycorrhizal growth benefit could mainly be attributed to an increase in the P and N uptake of the *M. sativa* plants (Fig. 2 and 3). The biomass of both root and shoot was positively correlated with the total P content in these tissues (Fig. 2a, c) (Table S8[8-9]), but not to the P tissue content per unit dry weight (later referred to as tissue concentration) (Fig. 2b, d). Plants that were colonized with high performance isolates had significantly higher root P contents than non-mycorrhizal controls or plants that were inoculated with the low performance isolates, but did not differ significantly from the medium performance isolates (Fig. 2a, Fig. S1, Table S3, Table S8[10-13]). In contrast, the P concentration in roots was negatively correlated to the biomass (Fig. 2b, Table S8[14]), but there were no significant differences in the P concentrations of the roots between the various isolate performance levels (Fig. S2, Table S3).

The correlation between shoot biomass and P content was not as strong as for roots (Fig. 2c, Table S8[9]). However, colonization with the high performance isolates (except *C. etunicatum* MG106) and several of the medium performance isolates led to an increase in the shoot P content relative to the non-mycorrhizal control plants (Table S8[15-18]). However, there were also several low performance isolates (*C. claroideum* UT159A*, R. irregulare* QB000, *P. occultum* CR102) that increased shoot P content compared to the controls (Fig. 2c, Fig. S1, Table S3). The shoot P tissue concentration was not correlated to the MGR, and plants inoculated with several of the low performance isolates had higher shoot P tissue concentrations than the non-mycorrhizal controls, or plants that were colonized with medium or high performance isolates (Fig. 2d, Fig. S2, Table S3, Table S8[20-23]).

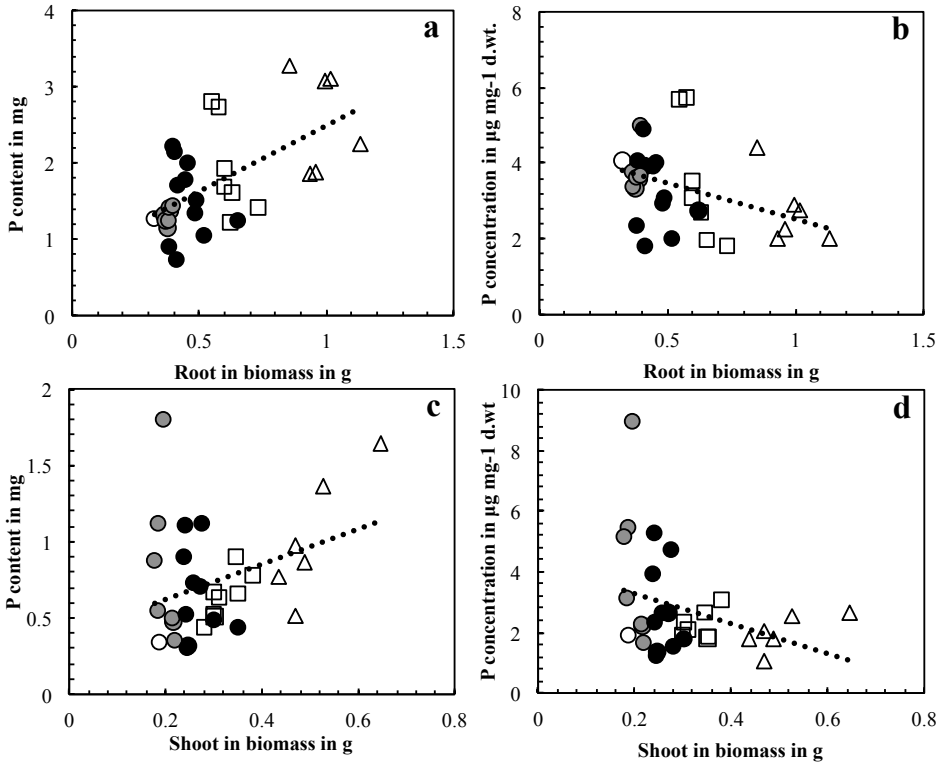

Fig. 2. Correlation between root (**a, b**) or shoot biomass (**c, d**) and P content (**a, c**) or concentration (**b, d**). Data of the non-mycorrhizal controls are shown as open circles, of plants inoculated with high performance isolates as open triangles, medium performance isolates as open squares, and low performance isolates as grey circles. All other fungal isolates that were not classified according to their symbiotic performance due to their high within-treatment variability are represented as black circles. Results of the regression analysis are as follows: (**a**) $r^2 = 0.6182$, $p = 0.0002$; (**b**) $r^2 = 0.157$, $p = 0.0247$; (**c**) $r^2 = 0.127$, $p = 0.045$; $r^2 = 0.119$, $p = 0.0531$.

Correlation between host biomass and N benefits of the AM symbiosis

There was a strong positive relationship between MGR and the effect of each fungal isolate on the N nutrition of the host. The growth of *M. sativa* was strongly positively correlated with both the total N content and tissue concentration of roots and shoots (Fig. 3, Fig. S3, S4, Table S9[1-4]). Plants that were colonized with the high performance isolates had significantly higher N contents and tissue concentrations in roots and shoots than those that were colonized with the low or medium performance isolates, or the non-mycorrhizal controls (Table S4, Table S9). The N tissue concentration of shoots of *M. sativa* colonized by high performance isolates was on average 211 % higher than in the non-mycorrhizal controls. Medium performance isolates only differed significantly in their effects on plant N contents or tissue concentrations from low performance isolates and non-mycorrhizal controls when they were combined in one performance group, but not when individual fungal isolates were compared (Table S4, Table S9[10-32]).

The effect of the fungal isolates on P and N nutrition and host biomass was not the result of differences in mycorrhizal colonization traits. Mycorrhizal performance was neither correlated to root colonization (Table S1), nor to the number of arbuscules per root length, nor to the length of the fungal ERM in the soil (Table S2), nor to spore number ($p > 0.05$). Only the estimated total arbuscular volume was positively correlated to the total plant biomass (Table S10[1]). Some of the plants had root nodules at harvest, but there was a high within treatment variability in root nodulation (0 to 4 biological replicates were nodulated), and the percentage of the root system that was nodulated was generally low (Table S2). Plant biomass and the N contents or concentrations in roots or shoots were not correlated to the extent of root nodulation (Table S10[2-8]).

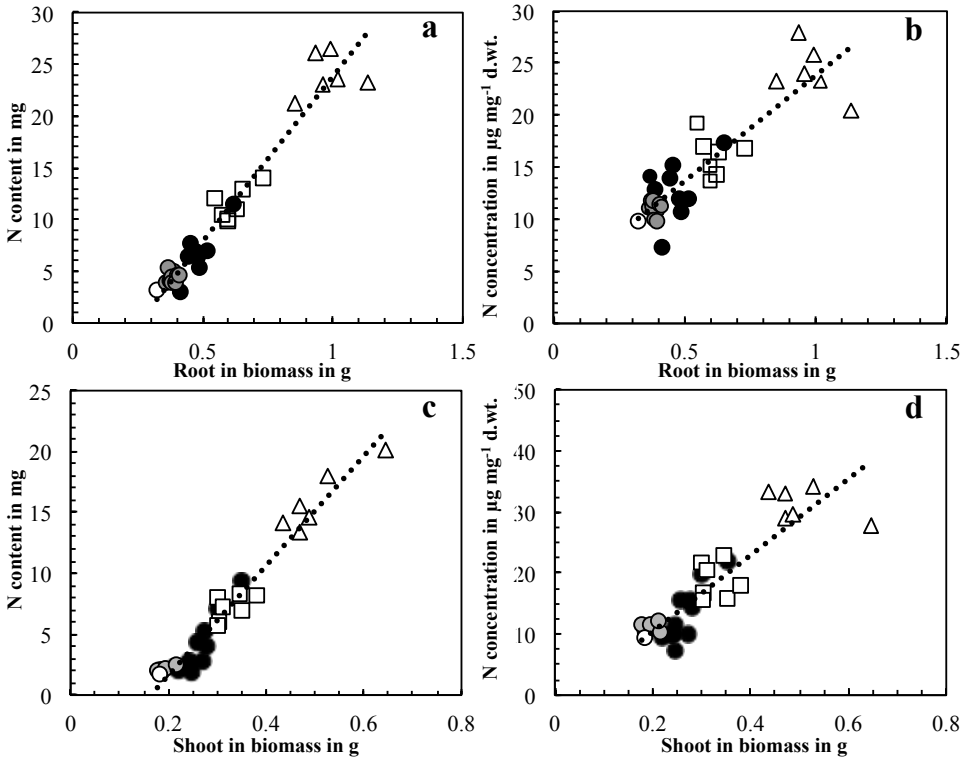

Fig. 3. Correlation between biomass of root (**a, b**) or shoot (**c, d**) and N content in mg (**a, c**) or N tissue concentration in µg mg^{-1} d.wt. (**b, d**). Data for the non-mycorrhizal controls are shown as open circles, of plants inoculated with high performance isolates as open triangles, medium performance isolates as open squares, and low performance isolates as grey circles. All other fungal isolates that were not classified according to their symbiotic performance due to their high within-treatment variability are represented as black circles. Results of the regression analysis are as follows: (**a**) $r^2 = 0.9494$, $p < 0.0001$; (**b**) $r^2 = 0.7917$, $p < 0.0001$; (**c**) $r^2 = 0.9511$, $p < 0.0001$; (**d**) $r^2 = 0.7833$, $p < 0.0001$.

Allocation of P in different P pools of the root

To determine whether the nutritional benefits conferred to *M. sativa* by the various fungal isolates were related to the P metabolism of the AM fungus, the % allocation of P in roots among different P pools was examined. DNA-P and lipid-P represented by far the largest P pools in the roots with on average 55.91 ± 1.3 % and 22.6 ± 0.76 %, respectively (Fig. S5b, e, Table S5). The P contents in these pools, which are largely related to host growth and biomass, were positively correlated to root biomass (Fig. S6b, e, Table S11[1, 2]). However, the tissue concentration or the percentage of P that was allocated to these pools did not differ significantly between roots colonized with high, medium or low performance isolates (Fig. S5b,e, Fig. S7b,e, Table S5, Table S6).

The metabolically active P_i pool (in %) in the roots was generally lower when plants were colonized with the high performance isolates (except *A. morrowiae* FL219B and *A. scrobiculata* VA104) than in plants that were colonized with the low performance isolates (Fig. S5a, Table S11[3-6]). The effects of the low performance isolates also differed significantly from that of medium performance isolates, when the isolates were grouped according to their performance, but not when individual isolates were compared (Table S11[5]). Root biomass was negatively correlated with the P_i tissue concentration in the root (Fig. 4a, Table S11[7]), and the P_i tissue concentrations in roots that were colonized with the high performance isolates, and several of the medium performance isolates, were generally lower than in roots that were colonized with the low performance isolates (Fig. S7a, Table S6, Table S11[9-10]). The P_i content in the roots that were colonized with the high performance isolates, however, did not differ significantly from the non-mycorrhizal controls, or those colonized with the low or medium performance isolates (Fig. S6a).

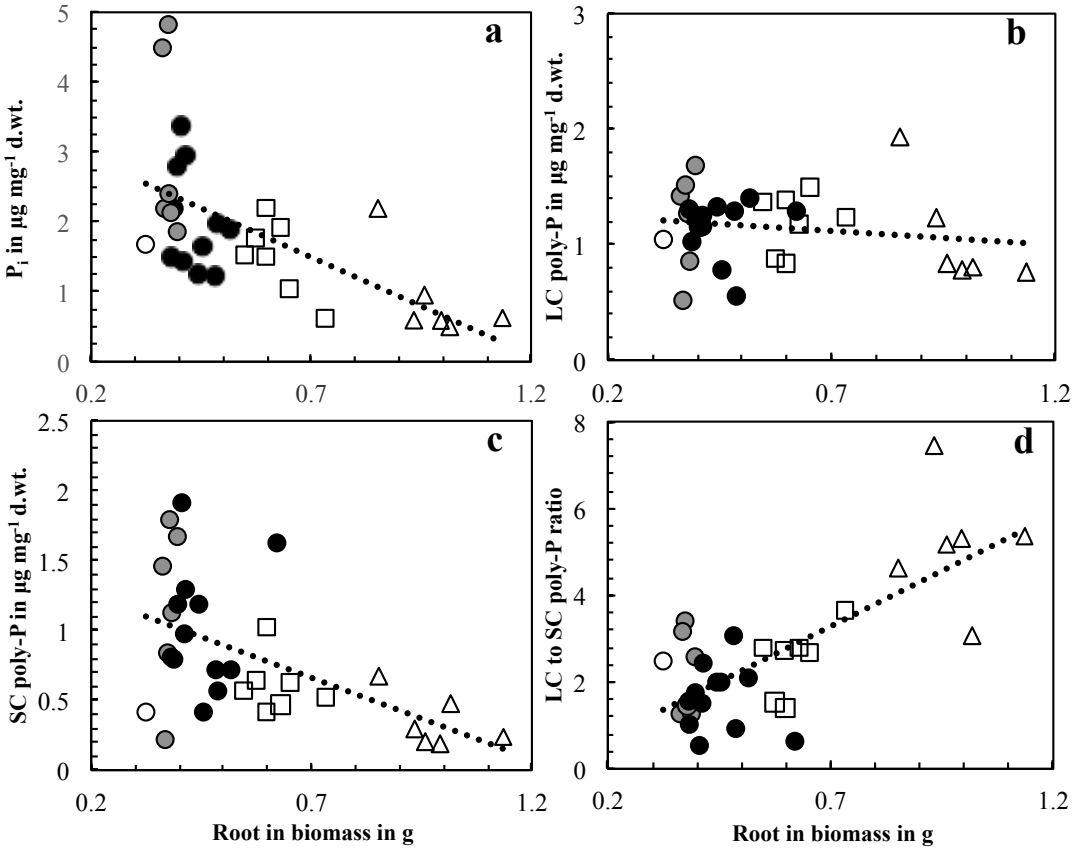

Fig. 4. Correlation between root biomass and tissue concentrations of P_i (**a**), long-chain poly-P (**b**), short-chain poly-P (**c**), and the ratio between long-chain and short-chain poly-P (**d**). Data of the non-mycorrhizal controls are shown as open circles, of plants inoculated with high performance isolates as open triangles, medium performance isolates as open squares, and low performance isolates as grey circles. All other fungal isolates that were not classified according to their symbiotic performance due to their high within-treatment variability are represented as black circles. Results of the regression analysis are as follows: (**a**) $r^2 = 0.1937$, $p < 0.0117$; (**b**) $r^2 = 0.029$, $p = 0.3494$; (**c**) $r^2 = 0.2953$, $p = 0.0013$; (**d**) $r^2 = 0.483$, $p < 0.0001$.

A large percentage of P in mycorrhizal roots was found in the poly-P pool. On average 11.04 ± 0.5 % (ranging from 4.0 to 20.3 %) of P in the roots was stored as long-chain or short-chain poly-P. This poly-P level was independent of the fungal identity (i.e. genus or morphospecies). Root biomass was not correlated with the total poly-P or long-chain poly-P pool, and the tissue concentration of long-chain poly-P in roots that were colonized with the high or the low performance isolates did not differ significantly (Fig. 4b, Fig. S5c, Fig. S7c). However, the content of long-chain poly-P in roots colonized with the high performance isolates was higher than in roots colonized with the low or medium performance isolates (Fig. S6c, Table S11[15-18]).

In contrast, the tissue concentration of short-chain poly-P was negatively correlated with the MGR (Fig. 4c, Fig. S7d, Table S11[19]). Similarly, when the fungal isolates were grouped according to their performance level, the tissue concentration of short-chain poly-P in roots colonized with the low performance isolates was significantly higher than in roots colonized with the medium or high performance isolates (Fig. S7d, Table S11[20-23]). However, when the isolates were compared individually, only *R. irregulare* QB000, *A. scrobiculata* CU130 and *A. columbiana* CL356 differed from five of the six high performance isolates (Table S6). The reduction in the concentration of short-chain poly-P tissue in the roots colonized with the high performance isolates changed the ratio between long- and short-chain poly-P in the roots; there was a clear positive correlation between MGR and an increase in the long-chain to short-chain poly-P ratio (Fig. 4d, Table S11[24]).

5.5 DISCUSSION

Approximately 200 different AM fungal morphospecies have been described so far, but the genetic and functional diversity among AM fungal strains is much larger than the small species number suggests (Koch *et al.,* 2006; Ehinger *et al.,* 2012). While it is appreciated that colonization by different isolates can lead to different host growth responses (Koch *et al.,* 2006; Ehinger *et al.,* 2012; Munkvold *et al.,* 2004), it is unknown what causes this high within species functional diversity. Here, the growth response of *M. sativa* was examined after colonization with 31 different fungal isolates from 10 morphospecies in to evaluate whether the poly-P metabolism in AM fungi is phylogenetically controlled, and whether differences in the efficiency with which AM fungi contribute to nutrient uptake and biomass development can be related to differences in P metabolism.

Based on the high variability in effects on the MGR among AM fungal isolates, the isolates were grouped into three performance levels. High performance isolates led in *M. sativa* to MGR of more than 170 %, medium performance isolates to MGR between 71 and 104 %, and low performance isolates did not lead to significant increases in plant biomass compared to the non-mycorrhizal controls (MGR $\leq$ 18 %). Fungal isolates within one performance level generally shared several important characteristics (e.g. their effect on P or N nutrition) under the present experimental conditions, and the performance levels were used to better describe these characteristics. However, MGR (or the performance level of an AM fungus) depends on the compatibility between the AM fungal symbiont and its host (Smith *et al.,* 2004), and is strongly context-dependent (Peng

et al., 1993). For example, the high performance isolates that were tested here led in *Achillea millefolium* L., and *Bromus inermis* Leyss to relatively low MGR, and in these plant species the intraspecific variability among the different fungal isolates was much less pronounced than in *M. sativa* (Koch *et al.,* unpublished).

Similar to the results of other authors (Avio *et al.,* 2009; Börstler *et al.,* 2008; Börstler *et al.,* 2010; Munkvold *et al.,* 2004), there was a high level of performance variability within a single AM fungal morphospecies, and many morphospecies included both high and low performance isolates. This high intraspecific variation is thought to contribute to the high phenotypic and functional diversity within AM fungal populations (Koch *et al.,* 2006). The high variability in MGR of *M. sativa* among isolates can be attributed to differences in the efficiency with which the various fungal isolates were able to contribute to the P and N nutrition of the host plant. Under the present experimental conditions, where it can be assumed that the availabilities of both P and N were growth-limiting, root and shoot biomass of *M. sativa* was positively correlated to the P and N content of these tissues, and to the tissue concentration of N in root and shoot.

However, MGR and high P and N levels of *M. sativa* were not related to any of the fungal growth and colonization patterns (Table S2, Koch *et al.,* unpublished). Fungal growth traits have been shown to be evolutionary conserved (Powell *et al.,* 2009), but the present results demonstrate that the effects of AM fungal isolates on host plant growth and P and N uptake are not conserved. This confirms the results of Munkvold *et al.,* (2004), who found that the length-specific hyphal P uptake is rather constant within one fungal species but that the within species variability in hyphal length, as well as effects on shoot growth response and shoot P content, are greater than the between species

variability, and that these functional characteristics are not aligned with the fungal phylogeny. This asymmetry indicates that the greater effect of some AM fungal isolates on plant P and N nutrition was more likely the result of more efficient P and N uptake systems and/or higher nutrient transport rates to the host. This is consistent with other studies in which no correlation between the dimensions of the ERM and P uptake and/or MGR was found (Hart & Reader 2002a; Smith *et al.,* 2000). A meta-analysis recently revealed that the mycorrhizal colonization is only in part responsible for the high diversity in MGR that can be observed, but that AM fungal taxa also differ in their mycorrhizal benefit per unit root length colonized (Treseder 2013). In contrast, in other reports, the functional diversity of AM fungal isolates was related to the dimensions or the interconnectedness of the ERM or to the absolute root length colonized (Avio *et al.,* 2006; Munkvold *et al.,* 2004). Similar to the results of Hart and Reader (2002a), who reported greater host benefits conferred by AM fungal families with larger internal mycelia, there was only a positive correlation between the total biomass of *M. sativa* and an estimate of the total arbuscular volume in the roots.

Several of the AM fungal isolates did not lead to significant biomass or nutritional gains in *M. sativa* compared to the non-mycorrhizal controls (neutral MGR). Neutral MGR have been observed under both non-limiting and growth-limiting levels of P in the soil (Smith & Smith 2013; Peng *et al.,* 1993). However, recent work suggests that AM fungi can also contribute to the P uptake of their host in the absence of positive MGR (Li *et al.,* 2006; Smith *et al.,* 2003). It has been suggested that negative or neutral MGR can be the result of a mycorrhiza-induced suppression of the plant P uptake pathway (via root hairs and epidermis) that is not compensated for by increases in the P uptake via the

mycorrhizal uptake pathway (via the ERM and the mycorrhizal interface) (Smith *et al.,* 2011; Smith & Smith 2011). There is evidence that AM fungi differ in their ability to inhibit the plant P uptake pathway. *R. intraradices*, for example, has been shown to nearly completely suppress the plant uptake pathway for P in several plant species, including *M. truncatula* (Smith *et al.,* 2004; Grunwald *et al.,* 2009). Of all the AM fungal species tested here, the four *Rhizophagus* isolates led to the lowest MGR (average of 20.2 ± 9.3 %) and the plants did not differ in their biomass from the non-mycorrhizal controls. However, the fact that the P tissue concentration in the shoot and the P_i level in the roots of plants that were colonized with *Rhizophagus*, and some of the other low performance isolates, were significantly higher than in the controls or plants that were colonized with several of the high performance isolates, could indicate that these fungi contributed to the P nutrition of the plants, despite their overall neutral MGR.

The high performance isolates significantly increased the P nutrition of *M. sativa* compared to the non-mycorrhizal controls and the low performance isolates. However, what really set these isolates apart from the non-mycorrhizal controls, and the low and medium performance isolates, was their positive impact on N nutrition. The N tissue concentration in the shoots of the plants that were colonized with the high performance isolates were on average 2.4 times higher, and the N content 3.8 times higher than in the non-mycorrhizal controls. While the positive effect of the AM symbiosis on P nutrition has been long known (Smith *et al.,* 2011; Smith & Read 2008), the role that AM fungi play in the N nutrition of their host is still under debate (for review see Smith & Smith 2011). It has been suggested that an improved N status of mycorrhizal plants may simply be a consequence of an improved P nutrition (Reynolds *et al.,* 2005). The present results,

however, suggest that the increase in the N nutrition of *M. sativa* by the high performance isolates was not only the result of an improved P nutrition, because both medium and high performance isolates increased the biomass of the plants and increased the P root contents compared to the controls. However, only the high performance isolates increased the N content of the plants and induced a greater biomass response than the medium performance isolates. These results confirm several other studies reporting a substantial contribution of AM fungi to the N nutrition of their host (Toussaint *et al.,* 2004; Tanaka & Yano 2005; Ngwene *et al.,* 2013; Nouri *et al.,* 2014).

The present work demonstrates that there is correlation between the nutritional benefits and the P metabolism of AM fungal isolates. The P_i and short-chain poly-P tissue concentrations in the root were negatively correlated, but the ratio between long-chain to short-chain poly-P was positively correlated to the root biomass. The P_i pool represents the metabolically active P pool. In plants and fungi, this pool is normally maintained at a constant level throughout a wide range of external supply conditions, and only severe P deficiency leads to a reduction in the P_i pool (Lee & Ratcliffe 1993; Robins & Ratcliffe 1984). The P_i levels in the roots that were colonized with the high performance isolates were not lower than those in the non-mycorrhizal controls, but reduced in comparison to the low performance isolates. It can be assumed that the reduced P_i levels in the roots colonized with the high performance isolates were caused by a dilution effect as a result of the high increase in plant biomass, rather than a symptom of P deficiency. This is also supported by the fact that the decrease in the P_i levels between these groups is consistent with the increase in plant biomass. This finding likewise supports our hypothesis that the high performance isolates differ from the medium performance isolates by their positive

effect on N nutrition, but that both groups of fungi contributed more or less equally to the P nutrition of their host.

The MGR of *M. sativa* was not correlated to the tissue concentration of long-chain poly-P in the roots. This suggests that the ability of medium and high performance isolates to provide P to the host was not the result of a reduced capacity of these fungi to store P as long-chain poly-P, and/or to a faster rate of remobilization of long-chain poly-P into short-chain poly-P. The constant tissue concentrations of long-chain poly-P in the roots, independent of fungal performance and plant biomass, seems to be more a reflection of the high P acquisition efficiency with which medium and high performance isolates are able to take up P from the soil.

The fact that low and high performance isolates did not differ in their effect on the long-chain poly-P concentration in roots, however, also indicates that low performance isolates still store a significant proportion of their available P as long-chain poly-P, despite the high P demand of their host and the presumably lower efficiency with which these fungi absorb P from the soil. The low efficiency with which the low performance isolates transferred P to their host could be the result of a low compatibility between the host and these fungal symbionts, but could also indicate that the low performance isolates still stored P in form of long-chain poly-P because the carbon supply from the host was low. The carbon supply from the host acts as an important trigger for P and N transport in the AM symbiosis (Fellbaum *et al.,* 2012b; Bücking & Shachar-Hill 2005; Hammer *et al.,* 2011; Fellbaum *et al.,* 2014), and it has been shown that both partners reciprocally reward partners that provide more mutualistic benefit (Kiers *et al.,* 2011). It can be assumed that the N and P supply levels in the present experiments were growth-limiting;

N deprivation will reduce the photosynthetic rates and will also limit the capability of the plant to provide carbon to its fungal symbionts (Konstantopoulou *et al.,* 2012). Medium and high performance isolates, on the other hand, may have been able to stimulate plant carbon supply by their positive impact on P and N nutrition, and consequently the photosynthetic efficiency of their host.

Poly-P play an important role for the P but also N transfer from the ERM to the IRM (Cruz *et al.,* 2007; Bücking & Shachar-Hill 2005; Ryan *et al.,* 2007; Viereck *et al.,* 2004). Consistently, the fungal isolates that contributed to both P and N nutrition of *M. sativa* showed the same characteristics in their P metabolism. It is generally hypothesized that long-chain poly-P are first broken down to short-chain poly-P, and subsequently remobilized by an exopolyphosphatase into P_i that can be transferred across the mycorrhizal interface (Ohtomo & Saito 2005). It has been suggested that long-chain poly-P better represent the long-term storage capacity of P in AM fungal hyphae, whereas short-chain poly-P are a good indicator of P transport to the host (Kiers *et al.,* 2011; Takanishi *et al.,* 2009). The present results seem to be contradictory to this view, because *M. sativa* roots colonized with high performance isolates had reduced levels of short-chain poly-P, and a high long-chain to short-chain poly-P ratio. This could indicate that medium and high performance isolates differ from low performance isolates in their capability to remobilize short-chain poly-P into P_i, but not in their capability to store P in form of long-chain poly-P. The particularly high long-chain to short-chain poly-P ratio in high performance isolates, however, also supports the view that medium and high performance isolates did not differ in their effect on P but in their effect on N nutrition. The high biomass of plants that were colonized with the high performance isolates would

first cause a dilution effect of the poly-P pool that is more readily available for the host, which supports the hypothesis that the short-chain poly-P pool is a good indicator for the P transport efficiency to the host (Takanishi *et al.,* 2009).

The majority of the AM fungal isolates used in this study were obtained from the International Culture Collection of Arbuscular Mycorrhizal Fungi (INVAM; http://invam.wvu.edu), and the isolates were renamed following the major taxonomic reclassification in the Glomeromycota (Schüßler & Walker 2010). However, since AM fungi belong to an ancient fungal lineage that has evolved for more than 500 million years without sexual reproduction, there is no good existing species concept (Corradi & Bonfante 2012). Traditionally, AM fungal species have been identified based on their spore morphology, but progress in molecular phylogeny has shown that spores with very similar morphologies can be produced by phylogenetically distant AM fungal species and several misclassified fungal morphospecies have recently been reclassified (Krüger *et al.,* 2012; Stockinger *et al.,* 2009). Due to the polymorphism within the rDNA, it has recently been estimated that the number of fungal species within the Glomeromycota is probably ten times larger than the small number of fungal morphospecies suggests (Buscot 2015). The high within species variability confirms that AM fungal morphospecies can differ greatly at the functional level.

It has recently been shown that even the genetic diversity in one spore can lead to genetically different variants, variable phenotypes and differences in MGR (Angelard *et al.,* 2010; Ehinger *et al.,* 2012). AM fungal growth traits have been shown to be phylogenetically conserved across the phylum Glomeromycota (Powell *et al.,* 2009) but based on the current classification of the morphospecies that were used in this study,

fungal effects on P or N nutrition were not phylogenetically conserved. In contrast, the present results demonstrate that the capability to contribute substantially to host plant benefit is wide spread across the phylum Glomeromycota. The asymmetry in conservatism between AM fungal traits and host plant performance suggests that the fungal adaptability to the host plant also plays an important role in the symbiotic performance of both partners (Smith *et al.,* 2004). This is also supported by the observation that the high performance strains did not consistently show the same symbiotic performance in other host plant species as in *M. sativa* (Koch *et al.,* unpublished).

In conclusion, mycorrhizal benefits are often discussed only in terms of an improved P nutrition and their respective carbon costs, but results here show that the plant growth response promoted by high performance isolates was related to their positive impact not only on P but also on N nutrition, and that the MGR was the result of the sum of these nutritional benefits (P and N) for the plant (Nouri *et al.,* 2014). It has been shown that P in combination with N limitation induces changes in the plant transcriptome that stimulate the AM colonization of plants under P and N stress despite an overall higher P status in mycorrhizal plants (Bonneau *et al.,* 2013). However, in addition to a high efficiency with which P and N are taken up, mycorrhizal growth benefits also depend on the rate with which fungal poly-P are remobilized and nutrients are released into the mycorrhizal interface. The high performance isolates examined here were particularly characterized by a high efficiency with which they took up P and N from the soil, but also by their capability with which they remobilized poly-P and released P and N in the IRM, and transferred these nutrients to their host. Considering the

key role that the P metabolism of the fungus plays for P and N transport in the symbiosis, it is crucial to better understand the physiological and regulatory mechanisms that contribute to the high functional diversity in P and N nutrition between the different AM fungal isolates. The results shown here only represent a snapshot of the P allocation into different P pools after 20 weeks of growth. Further experiments with P isotopes in time course experiments in multi-compartment systems are now necessary to track the P uptake by high and low performing isolates, and to follow the transport to the plant through the different P pools.

5.6. ACKNOWLEDGEMENTS

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5.8 SUPPLEMENTARY MATERIAL – FIGURES

HIGH FUNCTIONAL DIVERSITY WITHIN ARBUSCULAR MYCORRHIZAL FUNGAL SPECIES IS CORRELATED WITH DIFFERENCES IN PHOSPHATE AND NITROGEN UPTAKE AND FUNGAL PHOSPHATE METABOLISM

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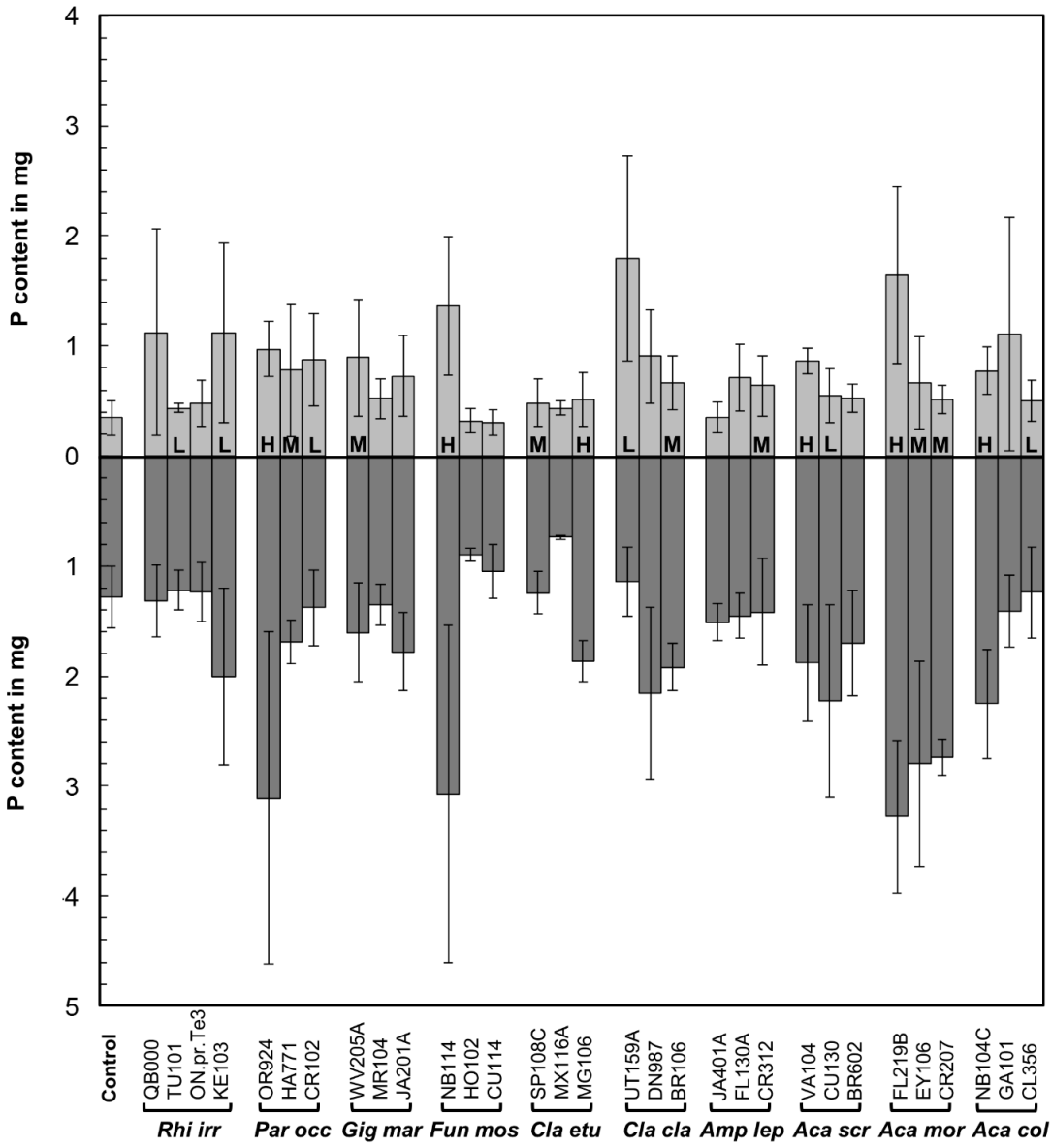

Fig. S1 Effect of different AM fungal isolates on the P contents in mg in root and shoot. The bars show means (n=4) of the P contents in mg of roots (bottom, dark grey) and shoots (top, light grey) and their respective confidence intervals ($p \leq 0.05$). The letters in the bars indicate whether the isolate belonged to the low (L), medium (M), or high (H) performance isolates.

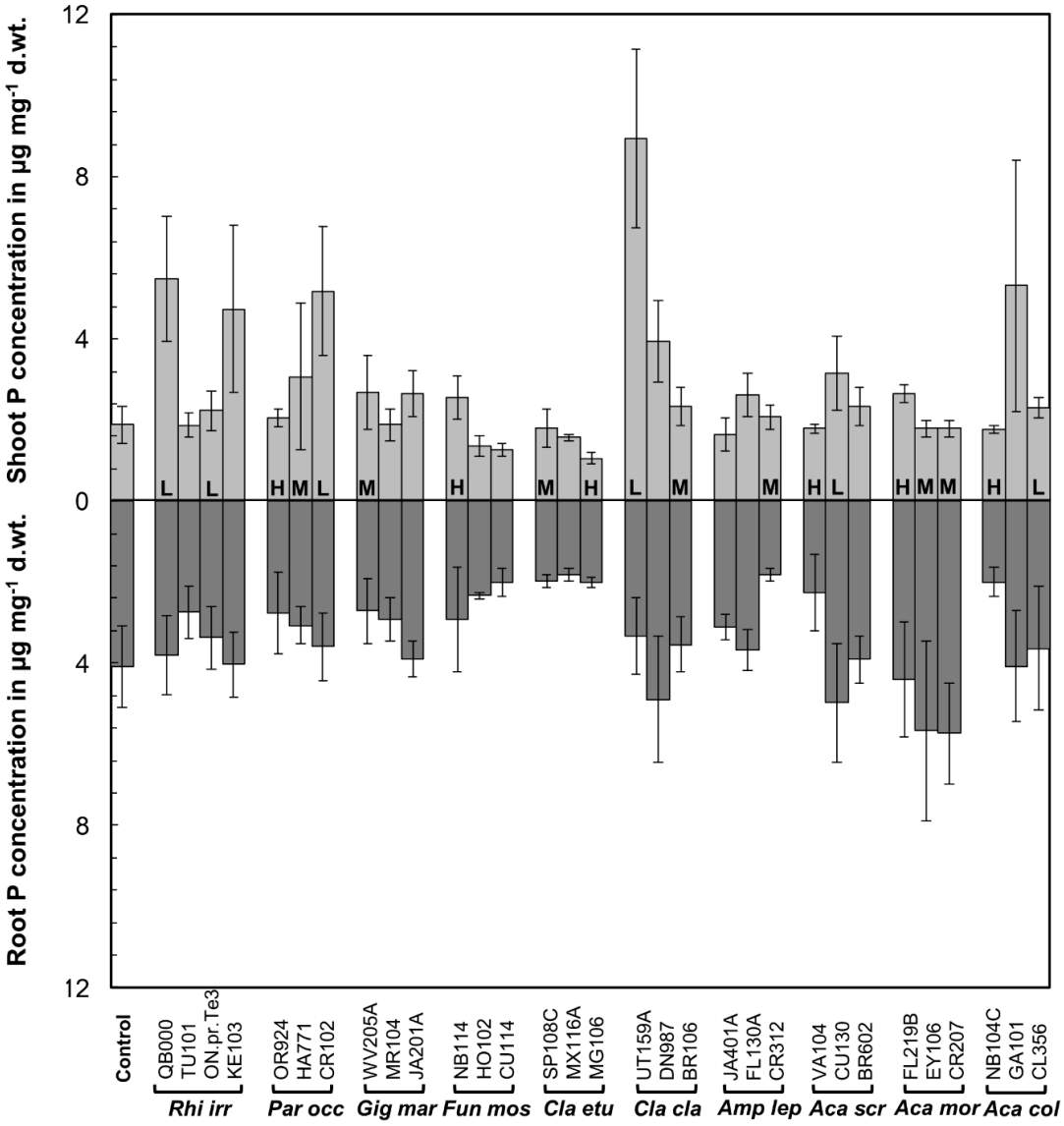

Fig. S2 Effect of different AM fungal isolates on the P tissue concentration in µg mg^{-1} d.wt. The bars show means (n=4) of the P tissue concentrations of roots (bottom, dark grey) and shoots (top, light grey) and their respective confidence intervals ($p \leq 0.05$). The letters in the bars indicate whether the isolate belonged to the low (L), medium (M), or high (H) performance isolates.

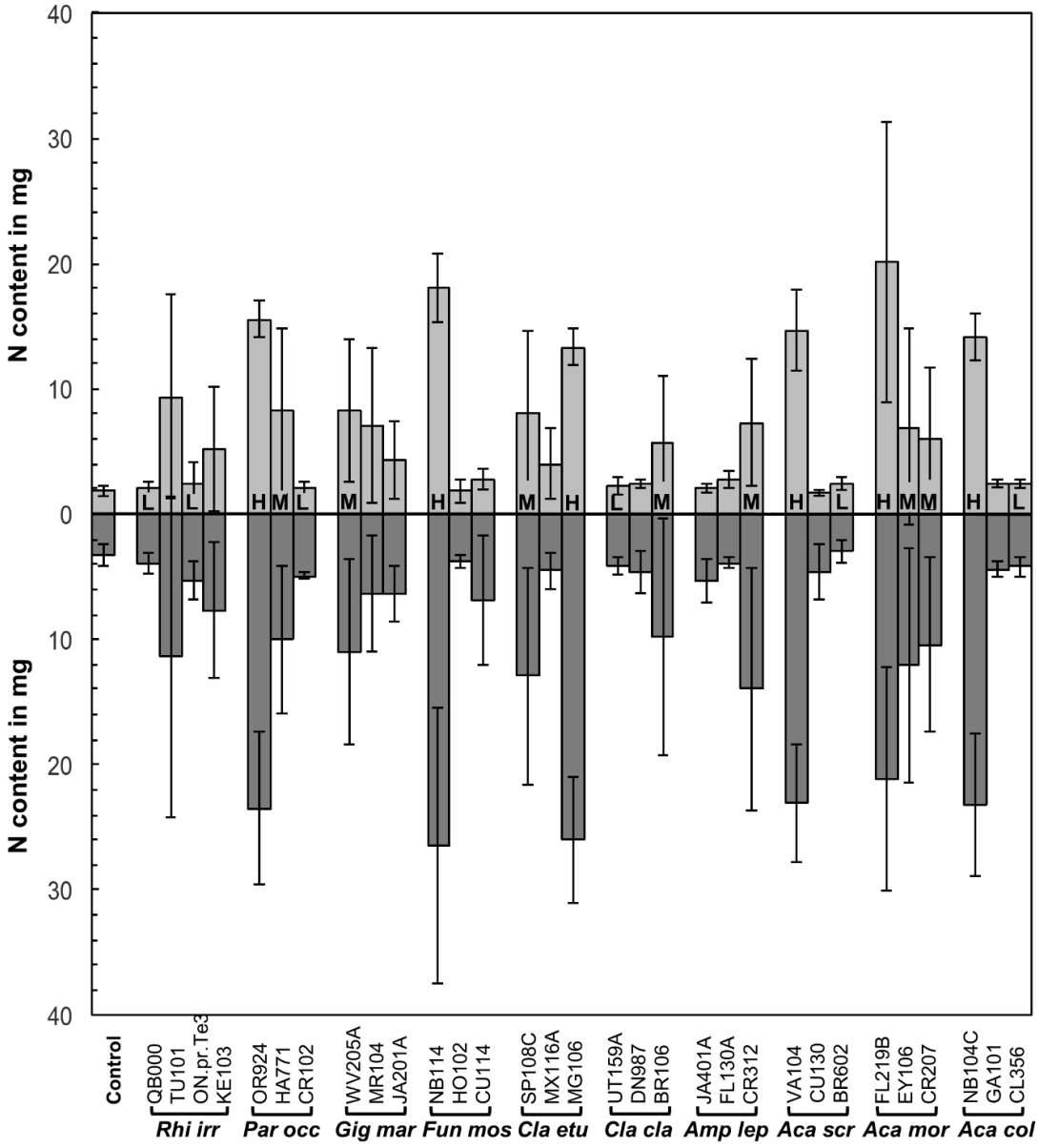

Fig. S3 Effect of different AM fungal isolates on the N contents in mg in root and shoot. The bars show means (n=4) of the N contents in mg of roots (bottom, dark grey) and shoots (top, light grey) and their respective confidence intervals ($p \leq 0.05$). The letters in the bars indicate whether the isolate belonged to the low (L), medium (M), or high (H) performance isolates.

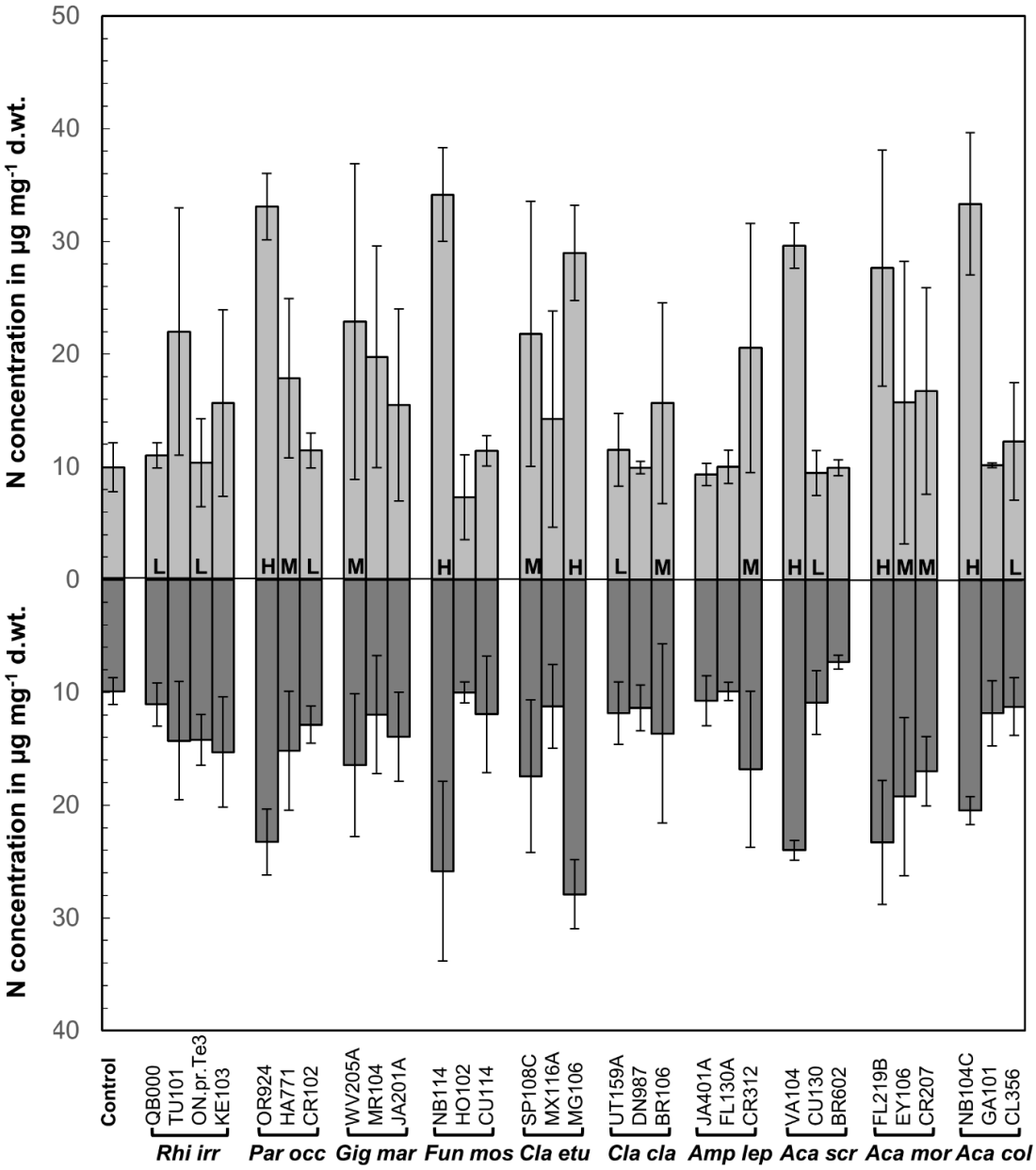

Fig. S4 Effect of different AM fungal isolates on the N tissue concentration in µg mg^{-1} d.wt. The bars show means (n=4) of the N tissue concentrations of roots (bottom, dark grey) and shoots (top, light grey) and their respective confidence intervals ($p \leq 0.05$). The letters in the bars indicate whether the isolate belonged to the low (L), medium (M), or high (H) performance isolates.

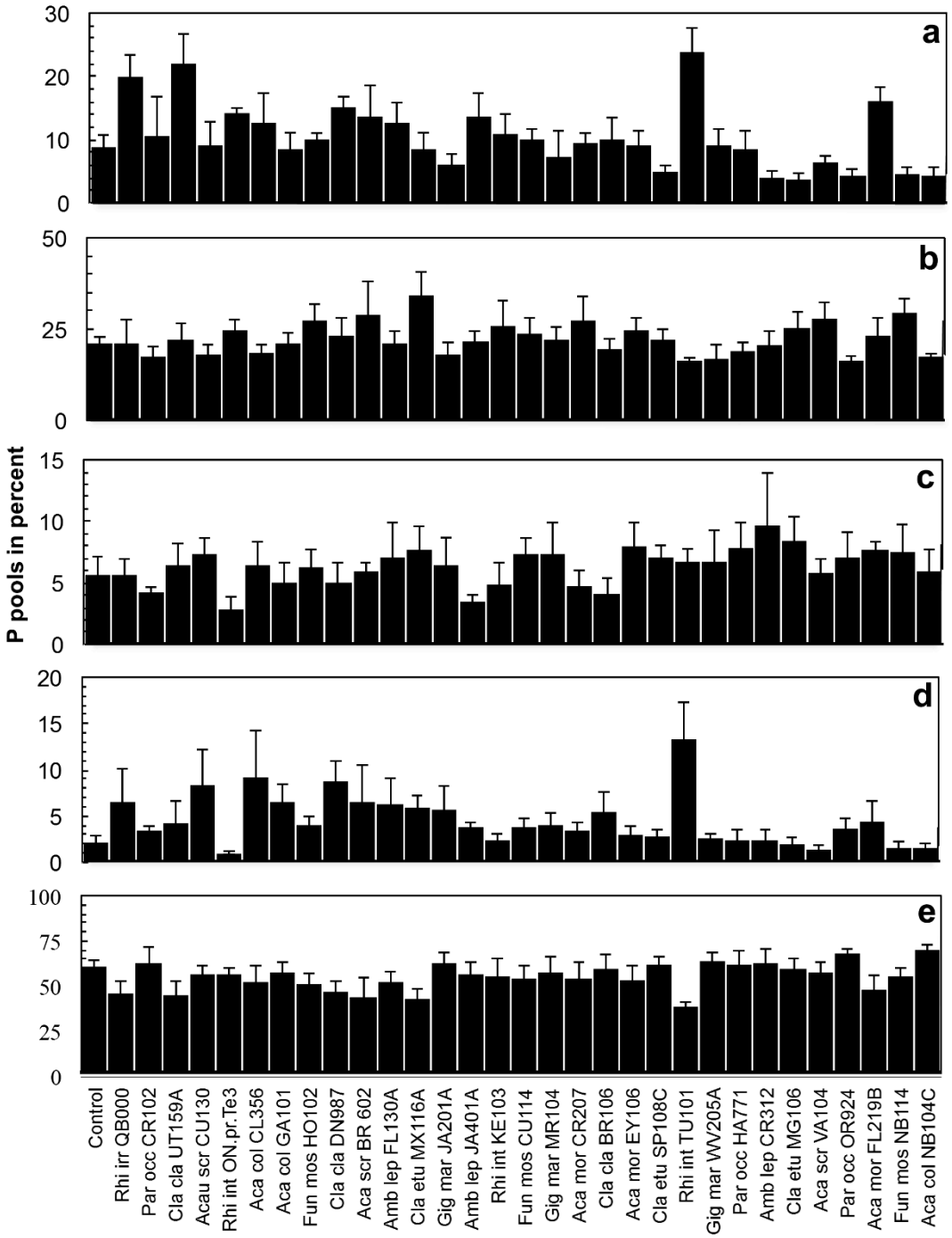

Fig. S5 Distribution of P in % between the metabolically active P_i pool (a), the lipid phosphate pool (b), the long-chained poly-P pool (c), the short-chained poly-P pool (d), and the DNA-, RNA-, and protein-phosphate pool (e). The controls and the different fungal isolates are given according to their MGR from left (lowest) to right (highest). The bars show means of n=4 and SEMs. Color code of the bars: high-performance isolates (white), medium performance isolates (middle grey), low performance isolates (light grey), all others and non-mycorrhizal controls (darkgrey).

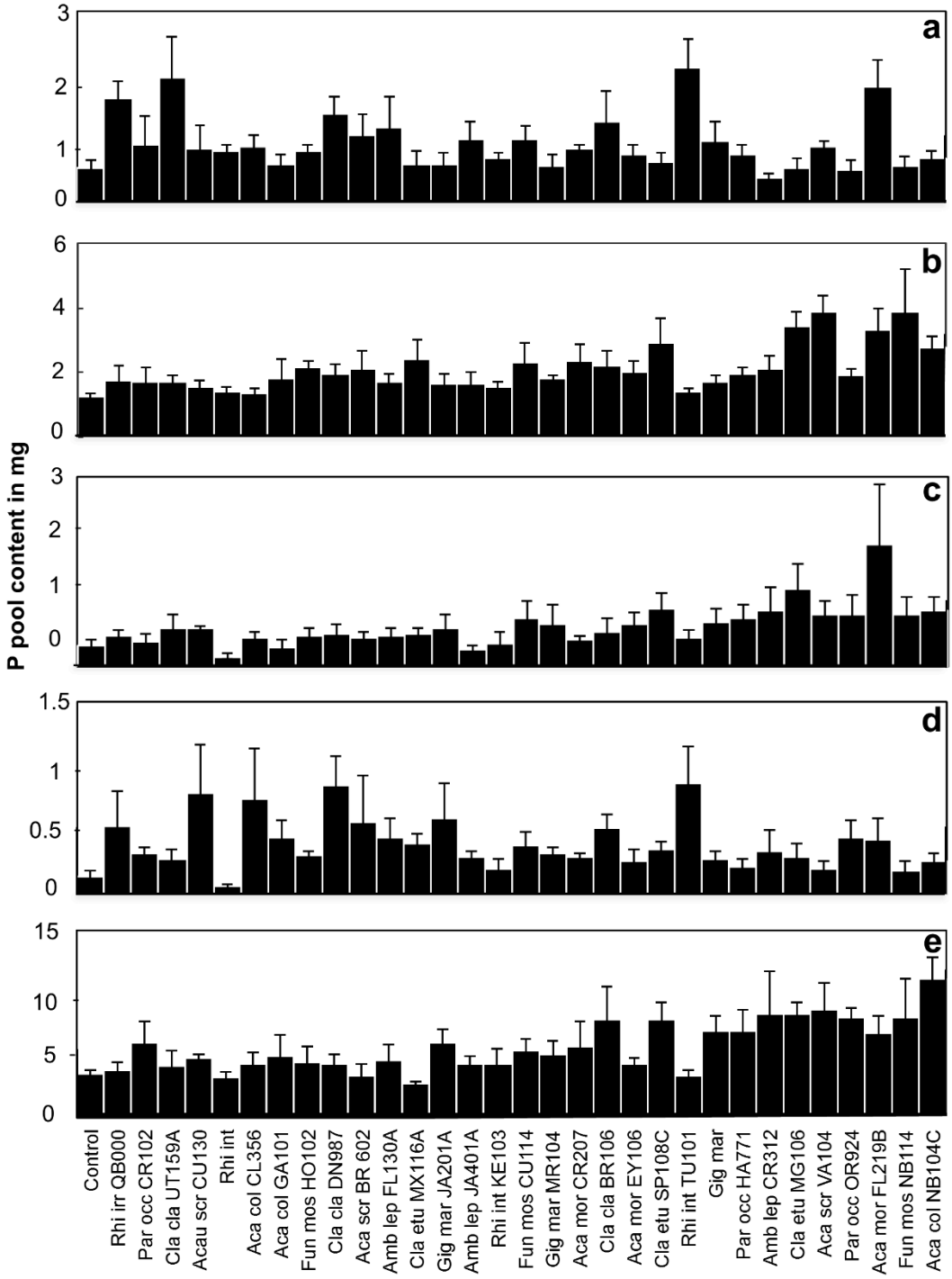

Fig. S6 P content in mg in the metabolically active P_i pool (a), the lipid phosphate pool (b), the long-chained poly-P pool (c), the short-chained poly-P pool (d), and the DNA-, RNA-, and protein-phosphate pool (e) of the roots. The controls and the different fungal isolates are given according to their MGR from left (lowest) to right (highest). The bars show means of n=4 and SEMs. Color code of the bars: high-performance isolates (white), medium performance isolates (middle grey), low performance isolates (light grey),all others and non-mycorrhizal controls (dark grey).

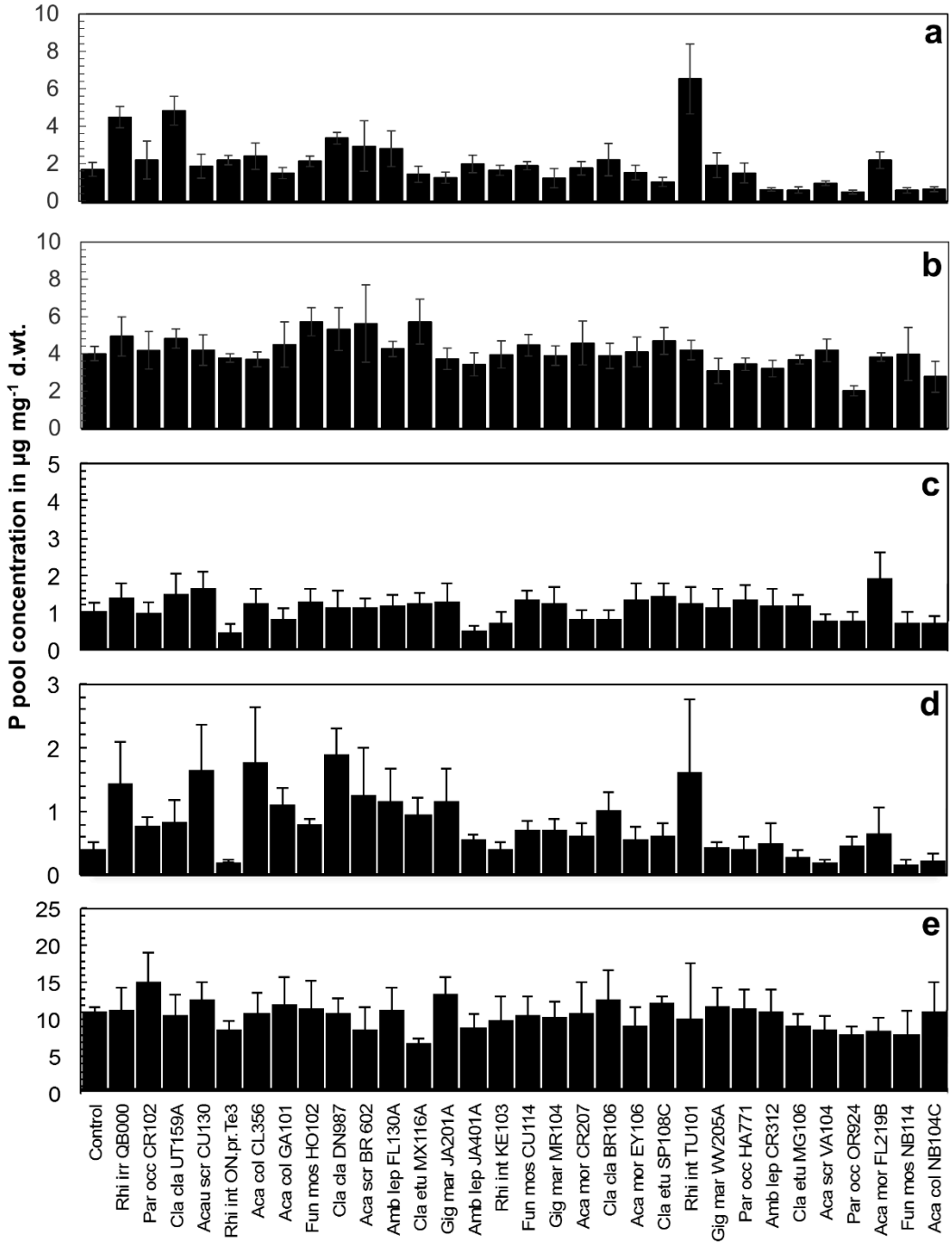

Fig. S7 P tissue concentration in µg mg^{-1} d.wt. in the metabolically active P_i pool (a), the lipid phosphate pool (b), the long-chained poly-P pool (c), the short-chained poly-P pool (d), and the DNA-, RNA-, and protein-phosphate pool (e) of the roots. The controls and the different fungal isolates are given according to their MGR from left (lowest) to right (highest). The bars show means of n=4 and SEMs. Color code of the bars: high-performance isolates (white), medium performance isolates (middle grey), low performance isolates (light grey), all others and non-mycorrhizal controls (dark grey).

5.9 SUPPLEMENTARY MATERIAL – TABLES

Table S1. Effect of the different AM fungal isolates on root, shoot, and total plant biomass, and mycorrhizal growth response (MGR). Isolates that were distinguished based on their performance level (PL) as low performance (LP), medium performance (MP) or high performance (HP) isolates are indicated. Data show the mean of n = 4 ± S.E.M. Different letters indicate statistically significant differences according to one-way ANOVA and LSD test ($p \leq 0.05$).

Fungal isolate	PL	Root biomass in mg		Shoot biomass in mg		Total plant biomass in mg		Colonization in %		Mycorrhizal growth response in %	
Controls		324.19 ± 30.5	h	186. ± 12.2	g	510.86 ± 28.06	g	1.4 ± 0.6	g	0.00 ± 5.49	g
Aca col CL356	LP	378.35 ± 31.0	gh	213. ± 23.6	fgh	591.97 ± 52.29	defg	34.1 ± 2.8	abc	15.88 ± 10.2	defg
Aca col GA101		383.29 ± 36.9	fgh	240. ± 21.2	efgh	623.61 ± 50.85	defg	34.6 ± 3.7	abc	22.07 ± 9.95	defg
Aca col NB104C	HP	1134.16 ± 150.	a	436. ± 51.3	bcd	1570.5 ± 185.9	a	27.8 ± 3.7	cd	207.44 ± 36.4	a
Acau mor CR207	MP	574.06 ± 154.	efg	303. ± 63.8	defgh	877.29 ± 218.5	cdef	37.0 ± 3.0	abc	71.73 ± 42.7	cdef
Acau mor EY106	MP	548.15 ± 128.	efgh	352. ± 75.7	cdef	900.15 ± 202.8	cdef	33.3 ± 2.2	abc	76.20 ± 39.7	cdef
Acau mor	HP	853.29 ± 136.	bcd	645. ± 172.	a	1498.7 ± 299.2	a	28.8 ± 1.3	c	193.39 ± 58.5	a
Acau scr BR 602		413.99 ± 77.5	fgh	242. ± 33.8	efgh	656.09 ± 109.5	cdefg	36.5 ± 2.5	abc	28.43 ± 21.4	cdefg
Acau scr CU130	LP	395.59 ± 62.6	fgh	183. ± 20.3	g	579.21 ± 68.31	efg	36.8 ± 2.4	abc	13.38 ± 13.3	efg
Acau scr VA104	HP	959.57 ± 113.	abc	488. ± 42.2	bc	1447.8 ± 128.8	a	29.4 ± 3.7	c	183.41 ± 25.2	a
Amb lep CR312	MP	731.57 ± 204.	cde	310. ± 58.9	defgh	1042.2 ± 263.0	bc	31.6 ± 2.3	abc	104.02 ± 51.4	bc
Amb lep FL130A		396.35 ± 28.6	fgh	271. ± 19.5	efgh	668.13 ± 44.51	cdefg	34.3 ± 3.6	abc	30.79 ± 8.71	cdefg
Amb lep JA401A		487.19 ± 35.8	efgh	218. ± 16.0	fgh	705.87 ± 34.95	cdefg	32.6 ± 2.1	abc	38.17 ± 6.84	cdefg
Cla cla BR106	MP	597.89 ± 128.	defg	301. ± 61.0	defgh	899.62 ± 189.1	cdef	42.0 ± 5.7	a	76.10 ± 37.0	cdef
Cla cla DN987		405.58 ± 61.9	fgh	237. ± 22.8	efgh	643.25 ± 60.60	defg	35.9 ± 3.1	abc	25.92 ± 11.8	defg
Cla cla UT159A	LP	375.06 ± 70.5	gh	195. ± 10.1	gh	570.14 ± 63.04	efg	32.6 ± 3.2	abc	11.60 ± 12.3	efg
Cla etu MG106	HP	933.33 ± 96.0	abc	470. ± 51.5	bc	1403.5 ± 107.6	ab	30.6 ± 2 5	bc	174.74 ± 21.0	ab
Cla etu MX116A		411.99 ± 37.2	fgh	279. ± 24.6	efgh	691.74 ± 59.86	cdefg	35.1 ± 3.2	abc	35.41 ± 11.7	cdefg
Cla etu SP108C	MP	652.34 ± 137.	def	300. ± 72.4	defgh	953.24 ± 208.8	cde	36.5 ± 5.2	abc	86.60 ± 40.8	cde
Fun mos CU114		517.79 ± 90.1	efgh	244. ± 38.9	efgh	762.24 ± 128.4	cdefg	32.6 ± 1.6	abc	49.21 ± 25.1	cdefg
Fun mos HO102		381.67 ± 12.9	fgh	246. ± 40.4	efgh	628.22 ± 36.29	defg	27.8 ± 1.2	cd	22.97 ± 7.10	defg
Fun mos NB114	HP	993.98 ± 69.0	abc	526. ± 23.9	ab	1520.7 ± 88.06	a	30.7 ± 4.7	bc	197.68 ± 17.2	a

Gig mar JA201A		444.18	±	43.8	fgh	257.	±	36.8	efgh	702.10	±	23.57	cdefg	16.9	±	5.1	ef	37.44	± 4.61 cdefg
Gig mar MR104		482.83	±	70.3	efgh	299.	±	66.1	defgh	782.15	±	134.0	cdefg	17.9	±	3.6	def	53.11	± 26.2 cdefg
Gig mar WV205A	MP	629.71	±	172.	defg	345.	±	56.0	cdefg	975.03	±	227.5	cd	9.5	±	3.2	fg	90.86	± 44.5 cd
Fungal isolate	PL	Root biomass in mg				Shoot biomass in mg				Total plant biomass in mg				Colonization in %				Mycorrhizal growth response in %	
Par occ CR102	LP	388.49	±	26.6	fgh	177.	±	18.5	g	566.14	±	40.29	efg	35.7	±	4.2	abc	10.82	± 7.89 efg
Par occ HA771	MP	596.55	±	111.	defg	380.	±	121.	bcde	977.43	±	229.4	cd	31.6	±	1.9	abc	91.33	± 44.9 cd
Par occ OR924	HP	1017.34	±	146.	ab	469.	±	13.4	bc	1487.0	±	136.1	a	34.9	±	3.6	abc	191.09	± 26.6 a
Rhi int KE103		455.37	±	106.	fgh	275.	±	63.0	efgh	730.42	±	166.0	cdefg	40.9	±	1.8	ab	42.98	± 32.5 cdefg
Rhi int ON.pr.Te3	LP	367.76	±	34.0	gh	216.	±	37.1	fgh	583.81	±	49.10	efg	27.8	±	1.2	abc	14.28	± 9.61 efg
Rhi int TU101		621.34	±	283.	gh	351.	±	108.	efgh	973.06	±	389.9	defg	36.4	±	3.3	abc	90.48	± 76.3 defg
Rhi irr QB000	LP	361.94	±	36.0	gh	186.	±	24.3	g	548.01	±	55.05	fg	36.6	±	3.3	cde	7.27	± 10.7 fg

Table S2. Colonization characteristics of the *Medicago sativa* roots. Shown is the d.wt. of the root nodules, the colonization of the roots with arbuscules, and vesicles, the hyphal length in the soil and an estimate of the arbuscular volume in the colonized roots. The performance level (PL) is based on the MGR and the isolates were distinguished into low performance (LP), medium performance (MP), and high performance (HP) fungi. Data are shown as mean of n = 4 ± S.E.M. Different letters indicate statistically significant differences according to one way ANOVA and LSD test ($p \leq 0.05$).

Fungal isolate	PL	Nodule d.wt. (mg)		Colonization with arbuscules (%)		Colonization with vesicles (%)		Hyphal length (m g^{-1} soil)		Arbuscular volume (mm^3)	
Controls		6.23 ± 6.23	cd	0.00 ± 0.00	e	0.00 ± 0.00	efghi	1.84 ± 0.46	f	0.00 ± 0.00	f
Aca col CL356	LP	0.00 ± 0.00	d	17.5 ± 3.77	cde	24.75 ± 5.91	abcd	4.13 ± 1.15	bcd	6.20 ± 1.65	def
Aca col GA101		39.77 ± 27.98	ab	29.7 ± 5.98	abcd	23.50 ± 6.29	abcd	3.25 ± 0.80	bcdef	11.66 ± 3.45	bcde
Aca col NB104C	HP	13.91 ± 2.65	bcd	29.0 ± 11.01	abcd	15.25 ± 1.49	d	4.03 ± 0.54	bcde	17.25 ± 6.80	abcde
Acau mor CR207	MP	5.00 ± 2.12	cd	33.0 ± 10.26	abc	28.75 ± 2.50	abc	4.08 ± 0.24	bcde	16.33 ± 5.98	abcde
Acau mor EY106	MP	28.18 ± 25.81	abcd	28.0 ± 6.36	abcd	32.50 ± 4.66	ab	3.88 ± 0.60	bcde	12.59 ± 4.10	bcde
Acau mor FL219B	HP	14.32 ± 7.03	bcd	30.2 ± 4.13	abcd	27.25 ± 4.73	abc	2.65 ± 0.42	cdef	15.97 ± 3.21	abcde
Acau scr BR 602		8.18 ± 8.18	bcd	34.7 ± 5.75	abc	33.00 ± 3.94	ab	2.45 ± 0.29	def	13.99 ± 3.75	abcde
Acau scr CU130	LP	6.59 ± 6.59	bcd	32.0 ± 7.15	abcd	29.75 ± 3.99	abc	3.88 ± 0.74	bcde	11.97 ± 2.97	bcde
Acau scr VA104	HP	12.5 ± 4.80	bcd	27.7 ± 6.57	abcd	25.00 ± 4.20	abcd	4.15 ± 1.06	bc	14.86 ± 3.52	abcde
Amb lep CR312	MP	1.36 ± 1.08	cd	32.5 ± 9.35	abc	24.00 ± 3.87	abcd	3.93 ± 0.77	bcde	17.85 ± 6.31	abcd
Amb lep FL130A		7.5 ± 7.5	bcd	32.5 ± 9.13	abc	28.50 ± 2.60	abc	3.23 ± 0.90	bcdef	13.49 ± 4.41	abcde
Amb lep JA401A		27.23 ± 15.79	abcd	27.0 ± 8.29	bcd	20.75 ± 2.78	cd	4.08 ± 0.57	bcde	11.85 ± 4.13	bcde
Cla cla BR106	MP	11.82 ± 11.52	bcd	48.0 ± 5.55	a	33.50 ± 4.21	a	2.40 ± 0.17	ef	25.12 ± 4.80	a
Cla cla DN987		2.27 ± 1.31	cd	34.7 ± 5.12	abc	21.50 ± 1.50	cd	4.28 ± 0.56	bc	14.16 ± 3.45	abcde
Cla cla UT159A	LP	14.32 ± 14.02	bcd	21.5 ± 3.80	cd	26.50 ± 5.39	abc	3.80 ± 0.48	bcde	7.54 ± 1.84	def
Cla etu MG106	HP	10.23 ± 1.83	bcd	26.7 ± 5.81	bcd	24.50 ± 6.02	abcd	3.98 ± 0.61	bcde	14.15 ± 3.11	abcde
Cla etu MX116A		58.86 ± 25.40	a	24.7 ± 6.21	bcd	34.00 ± 3.11	a	3.2 ± 0.32	bcdef	9.46 ± 2.87	cdef
Cla etu SP108C	MP	6.59 ± 4.02	bcd	34.5 ± 7.73	abc	31.00 ± 5.02	abc	3.80 ± 0.84	bcde	17.29 ± 5.14	abcde
Fun mos CU114		8.41 ± 5.13	bcd	26.5 ± 8.76	bcd	31.25 ± 2.50	abc	4.38 ± 0.66	b	11.88 ± 4.74	bcde
Fun mos HO102		5.22 ± 3.07	cd	26.7 ± 5.66	bcd	22.25 ± 2.87	bcd	2.78 ± 0.39	bcdef	9.87 ± 2.34	bcdef
Fun mos NB114	HP	7.95 ± 3.22	bcd	29.2 ± 9.67	abcd	28.50 ± 3.52	abc	4.43 ± 0.46	b	17.19 ± 6.31	abcde
Gig mar JA201A		0.23 ± 0.23	d	23.0 ± 8.35	cd	0.00 ± 0.00	e	6.63 ± 0.43	a	9.55 ± 3.48	cdef
Gig mar MR104		18.64 ± 11.95	bcd	23.7 ± 10.80	bcd	0.00 ± 0.00	e	3.73 ± 0.41	bcde	10.83 ± 5.23	bcdef
Gig mar WV205A	MP	11.59 ± 5.84	bcd	11.2 ± 4.96	de	0.00 ± 0.00	e	3.40 ± 0.66	bcde	5.20 ± 2.86	ef

Fungal isolate	PL	Nodule d.wt.	Colonization with arbuscules (%)	Colonization with vesicles (%)	Hyphal length (m g^{-1} soil)	Arbuscular volume (mm^3)
Par occ CR102	LP	16.59 ± 16.59 bcd	37.7 ± 11.36 abc	25.00 ± 6.20 abcd	3.60 ± 0.49 bcde	16.13 ± 5.48 abcde
Par occ HA771	MP	29.09 ± 10.79 abcd	29.5 ± 5.52 abcd	22.5 ± 5.30 bcd	4.20 ± 0.27 bc	13.85 ± 4.11 abcde
Par occ OR924	HP	18.86 ± 3.66 bcd	34.5 ± 10.28 abc	32.50 ± 3.80 ab	3.70 ± 0.38 bcde	21.32 ± 7.66 abc
Rhi int KE103		25.00 ± 19.17 bcd	35.5 ± 7.73 abc	33.00 ± 3.19 ab	3.13 ± 0.38 bcdef	15.66 ± 5.00 abcde
Rhi int ON.pr.Te3	LP	5.22 ± 5.22 cd	32.0 ± 8.50 abcd	25.25 ± 4.27 abcd	2.68 ± 0.73 cdef	12.37 ± 4.17 bcde
Rhi int TU101		21.36 ± 8.95 bcd	44.2 ± 5.95 ab	20.75 ± 5.39 cd	3.80 ± 0.71 bcde	22.21 ± 7.31 ab
Rhi irr QB000	LP	34.32 ± 21.71 abc	32.2 ± 8.00 abc	21.00 ± 1.68 cd	2.95 ± 0.59 bcdef	11.87 ± 3.06 bcde

Table S3. Effect of different fungal isolates on P root and shoot contents and tissue concentrations. Isolates that were distinguished based on their performance level (PL) as low performance (LP), medium performance (MP) or high performance (HP) isolates are indicated. Data show the mean of n = 4 ± S.E.M. Different letters indicate statistically significant differences according to one-way ANOVA and LSD test ($p \leq 0.05$).

Fungal isolate	PL	Root P concentration in µg mg^{-1} d.wt.	Root P content in mg	Shoot P concentration in µg mg^{-1} d.wt.	Shoot P content in mg
Controls		4.07 ± 1.01 abc	1.28 ± 0.28 cde	1.88 ± 0.46 def	0.34 ± 0.08 f
Aca col CL356	LP	3.63 ± 1.53 abc	1.24 ± 0.42 cde	2.31 ± 0.26 def	0.50 ± 0.09 def
Aca col GA101		4.07 ± 1.36 abc	1.42 ± 0.33 cde	5.31 ± 3.10 bc	1.10 ± 0.54 bcd
Aca col NB104C	HP	2.00 ± 0.36 c	2.25 ± 0.49 abcde	1.76 ± 0.09 ef	0.77 ± 0.11 cdef
Acau mor CR207	MP	5.73 ± 1.23 a	2.74 ± 0.16 abcd	1.79 ± 0.21 ef	0.51 ± 0.07 def
Acau mor EY106	MP	5.67 ± 2.22 a	2.80 ± 0.94 abc	1.79 ± 0.21 ef	0.66 ± 0.21 def
Acau mor FL219B	HP	4.41 ± 1.42 abc	3.28 ± 0.70 a	2.65 ± 0.22 cdef	1.64 ± 0.41 ab
Acau scr BR 602		3.91 ± 0.57 abc	1.70 ± 0.48 abcde	2.32 ± 0.46 def	0.53 ± 0.07 def
Acau scr CU130	LP	4.98 ± 1.47 ab	2.23 ± 0.87 abcde	3.16 ± 0.91 bcdef	0.55 ± 0.13 def
Acau scr VA104	HP	2.26 ± 0.94 bc	1.88 ± 0.53 abcde	1.78 ± 0.12 ef	0.86 ± 0.06 cdef
Amb lep CR312	MP	1.82 ± 0.14 c	1.42 ± 0.49 cde	2.07 ± 0.30 def	0.64 ± 0.14 defg
Amb lep FL130A		3.68 ± 0.50 abc	1.45 ± 0.21 cde	2.62 ± 0.52 cdef	0.71 ± 0.16 def
Amb lep JA401A		3.10 ± 0.31 abc	1.51 ± 0.17 bcde	1.64 ± 0.42 ef	0.35 ± 0.07 ef
Cla cla BR106	MP	3.54 ± 0.68 abc	1.92 ± 0.21 abcde	2.33 ± 0.48 def	0.66 ± 0.13 def
Cla cla DN987		4.89 ± 1.55 ab	2.16 ± 0.79 abcde	3.95 ± 1.00 bcde	0.91 ± 0.22 cdef
Cla cla UT159A	LP	3.33 ± 0.94 abc	1.14 ± 0.31 de	8.94 ± 2.20 a	1.80 ± 0.48 a
Cla etu MG106	HP	2.01 ± 0.12 c	1.86 ± 0.19 abcde	1.06 ± 0.14 f	0.52 ± 0.12 def
Cla etu MX116A		1.83 ± 0.16 c	0.74 ± 0.02 e	1.56 ± 0.08 ef	1.56 ± 0.03 ef
Cla etu SP108C	MP	1.98 ± 0.15 c	1.24 ± 0.19 cde	1.80 ± 0.47 ef	0.48 ± 0.11 def
Fun mos CU114		2.02 ± 0.35 c	1.05 ± 0.24 e	1.26 ± 0.14 ef	0.31 ± 0.06 f
Fun mos HO102		2.34 ± 0.07 bc	0.89 ± 0.06 e	1.36 ± 0.26 ef	0.32 ± 0.06 f
Fun mos NB114	HP	2.91 ± 1.29 bc	3.07 ± 1.53 ab	2.55 ± 0.53 cdef	1.36 ± 0.32 abc
Gig mar JA201A		3.90 ± 0.44 abc	1.78 ± 0.36 abcde	2.65 ± 0.56 cdef	0.73 ± 0.19 cdef
Gig mar MR104		2.93 ± 0.54 bc	1.35 ± 0.19 cde	1.88 ± 0.40 def	0.52 ± 0.09 def
Gig mar WV205A	MP	2.71 ± 0.80 bc	1.60 ± 0.45 bcde	2.67 ± 0.91 bcdef	0.90 ± 0.27 cdef

Fungal isolate	PL	Root P concentration in µg mg^{-1} d.wt.	Root P content in mg	Shoot P concentration in µg mg^{-1} d.wt.	Shoot P content in mg
Par occ CR102	LP	3.59 ± 0.83 abc	1.38 ± 0.34 cde	5.17 ± 1.59 bc	0.87 ± 0.21 cdef
Par occ HA771	MP	3.07 ± 0.46 abc	1.69 ± 0.20 abcde	3.07 ± 1.81 bcdef	0.78 ± 0.31 cdef
Par occ OR924	HP	2.77 ± 1.01 bc	3.11 ± 1.51 ab	2.06 ± 0.22 def	0.97 ± 0.13 cde
Rhi int KE103		4.03 ± 0.80 abc	2.00 ± 0.80 abcde	4.74 ± 2.06 bcd	1.11 ± 0.42 bcd
Rhi int	LP	3.37 ± 0.78 abc	1.23 ± 0.26 cde	2.23 ± 0.49 def	0.48 ± 0.11 def
Rhi int TU101		3.34 ± 0.38 abc	1.15 ± 0.24 cde	1.87 ± 0.34 def	0.43 ± 0.02 def
Rhi irr QB000	LP	3.79 ± 0.98 abc	1.32 ± 0.32 cde	5.48 ± 1.55 b	1.12 ± 0.48 bcd

Table S4. Effect of different fungal isolates on N root and shoot contents and tissue concentrations. Isolates that were distinguished based on their performance level (PL) as low performance (LP), medium performance (MP) or high performance (HP) isolates are indicated. Data show the mean of n = 4 ± S.E.M. Different letters indicate statistically significant differences according to one-way ANOVA and LSD test ($p \leq 0.05$).

Fungal isolate	PL	Root N concentration in µg mg^{-1} d.wt.		Root N content in mg		Shoot N concentration in µg mg^{-1} d.wt.		Shoot N content in mg	
Controls		9.90 ± 0.60	jk	3.24 ± 0.47	f	9.95 ± 1.11	efghi	1.85 ± 0.22	ef
Aca col CL356	LP	11.26 ± 1.31	ghijk	4.18 ± 0.42	def	12.27 ± 2.66	efghi	2.41 ± 0.20	ef
Aca col GA101		11.85 ± 1.46	ghijk	4.39 ± 0.34	def	10.15 ± 0.11	ghi	2.43 ± 0.17	ef
Aca col NB104C	HP	20.49 ± 0.63	bcde	23.26 ± 2.89	a	33.32 ± 3.22	ab	14.14 ± 0.96	abc
Acau mor CR207	MP	17.00 ± 1.57	defg	10.49 ± 3.55	cdef	16.73 ± 4.68	defghi	6.03 ± 2.87	ef
Acau mor EY106	MP	19.21 ± 3.58	bcdef	12.08 ± 4.78	cde	15.71 ± 6.39	efghi	6.96 ± 4.02	def
Acau mor	HP	23.28 ± 2.81	abcd	21.16 ± 4.54	ab	27.63 ± 5.35	abcd	20.07 ± 5.70	a
Acau scr BR 602		7.30 ± 0.32	k	2.99 ± 0.46	f	9.90 ± 0.36	hi	2.39 ± 0.29	ef
Acau scr CU130	LP	10.91 ± 1.45	ghijk	4.58 ± 1.13	def	9.45 ± 1.02	hi	1.69 ± 0.11	f
Acau scr VA104	HP	24.00 ± 0.45	abc	23.07 ± 2.40	a	29.63 ± 1.02	abc	14.61 ± 1.63	abc
Amb lep CR312	MP	16.80 ± 3.53	defgh	13.98 ± 4.95	bc	20.56 ± 5.64	cdefgh	7.24 ± 2.59	def
Amb lep FL130A		9.92 ± 0.40	ijk	3.92 ± 0.24	ef	9.99 ± 0.76	hi	2.76 ± 0.38	ef
Amb lep JA401A		10.73 ± 1.13	ghijk	5.33 ± 0.89	cdef	9.34 ± 0.50	hi	2.04 ± 0.17	ef
Cla cla BR106	MP	13.65 ± 4.06	efghijk	9.85 ± 4.84	cdef	15.66 ± 4.55	efghi	5.65 ± 2.72	ef
Cla cla DN987		11.38 ± 1.04	ghijk	4.64 ± 0.85	def	9.94 ± 0.28	hi	2.35 ± 0.18	ef
Cla cla UT159A	LP	11.84 ± 1.42	ghijk	4.13 ± 0.39	def	11.52 ± 1.65	efghi	2.25 ± 0.35	ef
Cla etu MG106	HP	27.91 ± 1.56	a	26.05 ± 2.55	a	28.98 ± 2.16	abc	13.31 ± 0.72	bcd
Cla etu MX116A		11.25 ± 1.90	ghijk	4.55 ± 0.74	def	14.24 ± 4.90	efghi	4.03 ± 1.47	ef
Cla etu SP108C	MP	17.43 ± 3.44	cdefg	12.93 ± 4.43	bcd	21.80 ± 6.01	bcdefg	8.04 ± 3.37	cdef
Fun mos CU114		11.94 ± 2.64	ghijk	6.95 ± 2.63	cdef	11.42 ± 0.68	efghi	2.78 ± 0.42	ef
Fun mos HO102		10.00 ± 0.48	hijk	3.83 ± 0.28	ef	7.31 ± 1.92	i	1.83 ± 0.49	ef
Fun mos NB114	HP	25.85 ± 4.07	ab	26.52 ± 5.63	a	34.15 ± 2.12	a	18.00 ± 1.39	ab
Gig mar JA201A		13.93 ± 2.02	efghijk	6.36 ± 1.14	cdef	15.50 ± 4.35	efghi	4.29 ± 1.57	ef
Gig mar MR104		11.96 ± 2.67	ghijk	6.41 ± 2.35	cdef	19.75 ± 5.02	cdefgh	7.05 ± 3.16	def
Gig mar	MP	16.45 ± 3.23	defghi	11.04 ± 3.79	cdef	22.90 ± 7.15	abcde	8.28 ± 2.90	cde

Fungal isolate	PL	Root N concentration in µg mg^{-1} d.wt.	Root N content in mg	Shoot N concentration in µg mg^{-1} d.wt.	Shoot N content in mg
Par occ CR102	LP	12.86 ± 0.84 fghijk	4.92 ± 0.12 def	11.46 ± 0.79 efghi	2.06 ± 0.30 ef
Par occ HA771	MP	15.18 ± 2.69 efghij	10.05 ± 3.00 cdef	17.88 ± 3.60 cdefghi	8.21 ± 3.38 cdef
Par occ OR924	HP	23.27 ± 1.49 abcd	23.50 ± 3.13 a	33.09 ± 1.50 ab	15.54 ± 0.76 ab
Rhi int KE103		15.28 ± 2.50 efghij	7.71 ± 2.77 cdef	15.67 ± 4.22 efghi	5.23 ± 2.55 ef
Rhi int ON.pr.Te3	LP	14.20 ± 1.16 efghij	5.31 ± 0.77 cdef	10.37 ± 2.00 fghi	2.47 ± 0.87 ef
Rhi int TU101		18.06 ± 5.88 ghijk	5.00 ± 2.25 def	11.34 ± 1.13 bcdef	3.92 ± 0.69 ef
Rhi irr QB000	LP	11.06 ± 0.98 ghijk	3.96 ± 0.41 def	11.01 ± 0.57 fghi	2.05 ± 0.25 ef

Table S5. P allocation in different P pools in roots in % . Effect of different fungal isolates on N root and shoot contents and tissue concentrations. Isolates that were distinguished based on their performance level (PL) as low performance (LP), medium performance (MP) or high performance (HP) isolates are indicated. Data show the mean of n = 4 ± S.E.M. Different letters indicate statistically significant differences according to one way ANOVA and LSD test ($p \leq 0.05$).

Fungal isolate	PL	Inorganic phosphate in %	Lipid-phosphate in %	Long-chained poly-P in %	Short-chained poly-P in %	DNA-, RNA-, and protein-phosphate in %
Controls		9.06 ± 1.85 defgh	21.52 ± 1.30 bcde	5.66 ± 1.39 bcd	2.30 ± 0.51 ef	61.45 ± 2.60 abcde
Aca col CL356	LP	12.88 ± 4.60 cdef	18.47 ± 2.17 cde	6.51 ± 1.86 bcd	± 4.87 ab	52.83 ± 8.05 bcdefg
Aca col GA101		8.70 ± 2.34 defgh	21.27 ± 2.44 bcde	5.08 ± 1.53 bcd	6.56 ± 1.87 bcd	58.39 ± 4.61 abcdef
Aca col NB104C	HP	4.57 ± 1.11 h	17.56 ± 0.72 de	6.02 ± 1.74 bcd	1.64 ± 0.51 ef	70.21 ± 2.87 a
Acau mor CR207	MP	9.59 ± 1.43 defgh	27.49 ± 6.61 abcde	4.82 ± 1.16 bcd	3.46 ± 0.91 cdef	54.64 ± 8.71 abcdef
Acau mor EY106	MP	9.39 ± 2.10 defgh	25.15 ± 3.06 abcde	8.09 ± 1.81 abcd	3.13 ± 0.71 def	54.23 ± 6.71 abcdef
Acau mor	HP	16.07 ± 2.21 cdef	23.24 ± 4.72 bcde	7.73 ± 0.59 a	4.60 ± 1.98 cdef	48.36 ± 7.17 cdefg
Acau scr BR 602		13.89 ± 4.72 cdef	28.94 ± 9.04 abc	6.02 ± 0.65 bcd	6.61 ± 3.83 bcd	44.53 ± 10.5 fg
Acau scr CU130	LP	9.22 ± 3.67 defgh	18.38 ± 2.40 cde	7.37 ± 1.34 abcd	8.37 ± 3.83 abc	56.66 ± 4.87 abcdef
Acau scr VA104	HP	6.47 ± 0.84 efgh	28.36 ± 4.15 abcd	5.93 ± 1.00 bcd	1.49 ± 0.39 ef	57.75 ± 5.60 abcdef
Amb lep CR312	MP	4.12 ± 0.92 h	20.76 ± 3.64 bcde	9.75 ± 4.15 ab	2.57 ± 1.04 ef	62.80 ± 7.63 abcd
Amb lep FL130A		12.77 ± 2.98 cdefg	21.47 ± 2.91 bcde	7.12 ± 2.82 abcd	6.40 ± 2.77 bcd	52.23 ± 5.78 bcdefg
Amb lep JA401A		13.82 ± 3.60 cdef	22.06 ± 2.34 bcde	3.48 ± 0.54 cd	3.84 ± 0.52 cdef	56.80 ± 6.40 abcdef
Cla cla BR106	MP	10.22 ± 3.17 defgh	19.74 ± 2.77 bcde	4.16 ± 1.30 cd	5.62 ± 1.99 bcd	60.26 ± 7.29 abcdef
Cla cla DN987		15.15 ± 1.76 bcd	23.24 ± 4.66 bcde	5.12 ± 1.49 bcd	8.81 ± 2.15 abc	47.69 ± 5.06 cdefg
Cla cla UT159A	LP	22.17 ± 4.50 ab	22.18 ± 4.16 bcde	6.45 ± 1.72 bcd	4.38 ± 2.15 bcd	44.82 ± 7.43 efg
Cla etu MG106	HP	3.82 ± 0.80 h	25.37 ± 4.22 abcde	8.52 ± 1.88 abc	2.08 ± 0.71 ef	60.23 ± 4.91 abcdef
Cla etu MX116A		8.64 ± 2.28 defgh	34.59 ± 5.92 a	7.78 ± 1.83 abcd	5.90 ± 1.26 bcd	43.09 ± 5.24 fg
Cla etu SP108C	MP	4.93 ± 1.04 gh	22.65 ± 2.26 bcde	7.17 ± 0.94 abcd	2.92 ± 0.67 ef	62.34 ± 4 abcd
Fun mos CU114		10.10 ± 1.62 defgh	23.81 ± 4.08 abcde	7.40 ± 1.30 abcd	3.87 ± 0.88 cdef	54.82 ± 5.83 abcdef
Fun mos HO102		10.27 ± 0.80 defgh	27.80 ± 3.84 abcde	6.39 ± 1.37 bcd	4.15 ± 0.74 bcd	51.39 ± 5.44 bcdefg
Fun mos NB114	HP	4.77 ± 0.78 h	29.89 ± 3.58 ab	7.63 ± 2.17 abcd	1.65 ± 0.53 ef	± 3.95 abcdef
Gig mar JA201A		6.22 ± 1.63 fgh	18.30 ± 3.12 cde	6.56 ± 2.08 bcd	5.70 ± 2.57 bcd	63.23 ± 5.61 abcd
Gig mar MR104		7.55 ± 3.72 defgh	22.37 ± 3.41 bcde	7.47 ± 2.41 abcd	4.16 ± 1.15 bcd	58.45 ± 7.50 abcdef
Gig mar	MP	9.35 ± 2.45 defgh	17.26 ± 3.49 e	6.76 ± 2.53 bcd	2.65 ± 0.38 ef	63.98 ± 4.30 abc

Fungal isolate	PL	Inorganic phosphate in %	Lipid-phosphate in %	Long-chained poly-P in %	Short-chained poly-P in %	DNA-, RNA-, and protein-phosphate in %
Par occ CR102	LP	10.90 ± 5.86 defgh	17.53 ± 2.76 de	4.32 ± 0.24 bcd	3.56 ± 0.33 cdef	± 8.20 abc
Par occ HA771	MP	8.51 ± 2.88 defgh	19.20 ± 2.09 bcde	7.87 ± 1.97 abcd	2.38 ± 1.03 ef	62.04 ± 7.05 abcde
Par occ OR924	HP	4.31 ± 1.00 h	16.60 ± 0.84 e	7.07 ± 2.08 abcd	3.74 ± 0.95 cdef	68.28 ± 2.60 ab
Rhi int KE103		10.92 ± 3.19 defgh	25.84 ± 6.96 abcde	5.02 ± 1.66 bcd	2.48 ± 0.58 ef	55.74 ± 9.37 abcdef
Rhi int	LP	14.23 ± 0.84 cde	25.06 ± 2.51 abcde	2.94 ± 0.97 d	1.08 ± 0.21 f	56.70 ± 2.97 abcdef
Rhi int TU101		24.00 ± 3.57 a	16.38 ± 0.64 e	6.83 ± 0.96 abcd	13.46 ± 3.88 a	39.33 ± 2.12 g
Rhi irr QB000	LP	19.97 ± 3.45 abc	21.60 ± 5.85 bcde	5.74 ± 1.23 bcd	6.58 ± 3.42 bcd	46.11 ± 6.93 defg

Table S6. P allocation in different P pools in µg per mg^{-1} d.wt. (tissue concentrations). Isolates that were distinguished based on their performance level (PL) as low performance (LP), medium performance (MP) or high performance (HP) isolates are indicated. Data show the mean of n = 4 ± S.E.M. Different letters indicate statistically significant differences according to one-way ANOVA and LSD test ($p \leq 0.05$).

Fungal isolate	PL	Inorganic phosphate in µg mg^{-1} d.wt.	Lipid-phosphate in µg mg^{-1} d.wt.	Long-chained poly-P in µg mg^{-1} d.wt.	Short-chained poly-P in µg mg^{-1} d.wt.	DNA, RNA, Prot.-P in µg mg^{-1} d.wt.
Controls		1.69 ± 0.36 efghi	4.00 ± 0.39 abcd	1.04 ± 0.23 abc	0.41 ± 0.08 fgh	11.26 ± 0.44 ab
Aca col CL356	LP	2.41 ± 0.70 defg	3.70 ± 0.40 abcd	1.28 ± 0.33 abc	1.80 ± 0.85 ab	11.04 ± 2.45 ab
Aca col GA101		1.50 ± 0.29 efghi	4.49 ± 1.21 abc	0.85 ± 0.25 bc	1.12 ± 0.26 abcdef	12.29 ± 3.56 ab
Aca col NB104C	HP	0.63 ± 0.12 hi	2.78 ± 0.83 cd	0.76 ± 0.15 bc	0.24 ± 0.08 gh	11.33 ± 3.80 ab
Acau mor CR207	MP	1.77 ± 0.36 defghi	4.57 ± 1.18 abc	0.88 ± 0.19 bc	0.64 ± 0.18 defgh	11.04 ± 3.98 ab
Acau mor EY106	MP	1.52 ± 0.39 efghi	4.11 ± 0.79 abcd	1.37 ± 0.40 abc	0.57 ± 0.18 efgh	9.20 ± 2.55 ab
Acau mor FL219B	HP	2.19 ± 0.44 defgh	3.81 ± 0.23 abcd	1.93 ± 0.67 a	0.67 ± 0.40 defgh	8.60 ± 1.67 ab
Acau scr BR 602		2.94 ± 1.34 cde	5.62 ± 2.08 a	1.15 ± 0.23 abc	1.29 ± 0.71 abcdef	8.93 ± 2.84 ab
Acau scr CU130	LP	1.86 ± 0.64 defghi	4.19 ± 0.83 abcd	1.68 ± 0.44 ab	1.67 ± 0.71 abc	12.87 ± 2.05 ab
Acau scr VA104	HP	0.95 ± 0.12 ghi	4.19 ± 0.60 abcd	0.83 ± 0.11 bc	0.20 ± 0.04 h	8.81 ± 1.75 ab
Amb lep CR312	MP	0.62 ± 0.09 hi	3.20 ± 0.45 bcd	1.24 ± 0.41 abc	0.52 ± 0.28 efgh	11.18 ± 2.99 ab
Amb lep FL130A		2.79 ± 0.96 def	4.26 ± 0.40 abcd	1.24 ± 0.26 abc	1.19 ± 0.47 abcdef	11.48 ± 2.90 ab
Amb lep JA401A		2.00 ± 0.47 defghi	3.42 ± 0.62 abcd	0.55 ± 0.12 c	0.57 ± 0.05 efgh	8.97 ± 1.85 ab
Cla cla BR106	MP	2.21 ± 0.87 defgh	3.89 ± 0.68 abcd	0.83 ± 0.24 bc	1.03 ± 0.27 abcdef	12.84 ± 3.76 ab
Cla cla DN987		3.36 ± 0.31 bcd	5.31 ± 1.15 ab	1.16 ± 0.39 abc	1.91 ± 0.41 a	11.08 ± 1.91 ab
Cla cla UT159A	LP	4.83 ± 0.77 ab	4.81 ± 0.52 abc	1.51 ± 0.53 ab	0.84 ± 0.34 bcdefg	10.71 ± 2.76 ab
Cla etu MG106	HP	0.60 ± 0.17 hi	3.68 ± 0.24 abcd	1.23 ± 0.26 abc	0.30 ± 0.09 gh	9.31 ± 1.48 ab
Cla etu MX116A		1.44 ± 0.43 efghi	5.71 ± 1.20 a	1.25 ± 0.30 abc	0.98 ± 0.23 abcdef	6.83 ± 0.47 b
Cla etu SP108C	MP	1.03 ± 0.25 ghi	4.69 ± 0.73 abc	1.49 ± 0.29 ab	0.62 ± 0.19 defgh	12.49 ± 0.63 ab
Fun mos CU114		1.89 ± 0.22 defghi	4.46 ± 0.58 abc	1.40 ± 0.19 abc	0.72 ± 0.13 cdefgh	10.84 ± 2.17 ab
Fun mos HO102		2.13 ± 0.28 defghi	5.72 ± 0.76 a	1.31 ± 0.32 abc	0.81 ± 0.08 cdefgh	11.77 ± 3.61 ab
Fun mos NB114	HP	0.58 ± 0.14 hi	3.98 ± 1.43 abcd	0.78 ± 0.21 bc	0.18 ± 0.07 h	8.18 ± 3.11 b
Gig mar JA201A		1.26 ± 0.29 fghi	3.72 ± 0.57 abcd	1.33 ± 0.46 abc	1.18 ± 0.48 abcdef	13.53 ± 2.30 ab
Gig mar MR104		1.23 ± 0.52 fghi	3.90 ± 0.51 abcd	1.29 ± 0.37 abc	0.72 ± 0.17 cdefgh	10.56 ± 1.85 ab
Gig mar WV205A	MP	1.92 ± 0.67 defghi	3.08 ± 0.66 bcd	1.18 ± 0.45 abc	0.46 ± 0.05 fgh	11.93 ± 2.31 ab

Fungal isolate	PL	Inorganic phosphate in µg mg^{-1} d.wt.	Lipid-phosphate in µg mg^{-1} d.wt.	Long-chained poly-P in µg mg^{-1} d.wt.	Short-chained poly-P in µg mg^{-1} d.wt.	DNA, RNA, Prot.-P in µg mg^{-1} d.wt.
Par occ CR102	LP	2.19 ± 1.01 defgh	4.18 ± 1.00 abcd	1.03 ± 0.22 abc	0.80 ± 0.11 cdefgh	15.28 ± 3.90 a
Par occ HA771	MP	1.51 ± 0.54 efghi	3.44 ± 0.34 abcd	1.39 ± 0.33 abc	0.42 ± 0.20 fgh	11.68 ± 2.29 ab
Par occ OR924	HP	0.49 ± 0.09 i	2.00 ± 0.27 d	0.81 ± 0.21 bc	0.47 ± 0.13 fgh	8.16 ± 0.91 b
Rhi int KE103		1.64 ± 0.27 efghi	3.96 ± 0.73 abcd	0.77 ± 0.25 bc	0.41 ± 0.09 fgh	10.06 ± 3.06 ab
Rhi int ON.pr.Te3	LP	2.19 ± 0.24 defgh	3.78 ± 0.22 abcd	0.52 ± 0.18 c	0.21 ± 0.02 h	8.76 ± 0.93 ab
Rhi int TU101		5.19 ± 2.03 a	4.85 ± 0.77 abcd	1.71 ± 0.48 ab	1.62 ± 1.12 abcd	10.26 ± 8.64 ab
Rhi irr QB000	LP	4.49 ± 0.56 bc	4.94 ± 1.05 abc	1.41 ± 0.39 abc	1.46 ± 0.65 abcde	11.48 ± 2.91 ab

Table S7. P allocation in different P pools in mg per root (contents). Isolates that were distinguished based on their performance level (PL) as low performance (LP), medium performance (MP) or high performance (HP) isolates are indicated. Data show the mean of n = 4 ± S.E.M. Different letters indicate statistically significant differences according to one-way ANOVA and LSD test ($p \leq 0.05$).

Fungal isolate	PL	Inorganic phosphate in mg	Lipid-phosphate in mg	Long-chained poly-P in mg	Short-chained poly-P in mg	DNA-, RNA-, protein-phosphate in mg
Controls		0.54 ± 0.13 fg	1.26 ± 0.10 g	0.35 ± 0.09 d	0.14 ± 0.04 fg	3.60 ± 0.26 fgh
Aca col CL356	LP	0.87 ± 0.20 defg	1.37 ± 0.13 fg	0.45 ± 0.11 d	0.75 ± 0.39 abcd	4.23 ± 1.03 defgh
Aca col GA101		0.60 ± 0.16 fg	1.83 ± 0.57 defg	0.32 ± 0.10 d	0.44 ± 0.13 bcdefg	4.93 ± 1.64 bcdefg
Aca col NB104C	HP	0.69 ± 0.13 efg	2.77 ± 0.34 abcde	0.88 ± 0.23 cd	0.25 ± 0.07 efg	11.16 ± 1.63 a
Acau mor CR207	MP	0.85 ± 0.05 efg	2.35 ± 0.54 bcdefg	0.42 ± 0.61 d	0.29 ± 0.03 efg	5.75 ± 2.03 bcdefg
Acau mor EY106	MP	0.75 ± 0.16 efg	2.03 ± 0.34 defg	0.67 ± 0.19 cd	0.26 ± 0.08 efg	4.30 ± 0.48 defgh
Acau mor FL219B	HP	1.80 ± 0.45 abc	3.33 ± 0.65 abc	1.94 ± 0.95 b	0.42 ± 0.17 bcdefg	6.83 ± 1.28 bcdef
Acau scr BR 602		1.05 ± 0.34 cdefg	2.09 ± 0.58 cdefg	0.46 ± 0.07 cd	0.56 ± 0.36 bcde	3.41 ± 0.83 fgh
Acau scr CU130	LP	0.85 ± 0.36 efg	1.55 ± 0.23 efg	0.60 ± 0.05 cd	0.79 ± 0.38 abc	4.72 ± 0.33 bcdefg
Acau scr VA104	HP	0.89 ± 0.08 defg	3.91 ± 0.50 a	0.84 ± 0.19 cd	0.20 ± 0.06 efg	8.67 ± 2.18 ab
Amb lep CR312	MP	0.40 ± 0.05 g	2.13 ± 0.39 cdefg	0.89 ± 0.35 cd	0.34 ± 0.16 cdefgg	8.26 ± 3.47 abcd
Amb lep FL130A		1.17 ± 0.49 cdef	1.71 ± 0.25 defg	0.48 ± 0.12 cd	0.44 ± 0.15 bcdefg	4.57 ± 1.21 cdefgh
Amb lep JA401A		1.00 ± 0.27 defg	1.67 ± 0.32 efg	0.27 ± 0.06 d	0.28 ± 0.05 efg	4.27 ± 0.72 defgh
Cla cla BR106	MP	1.25 ± 0.50 bcdef	2.23 ± 0.45 bcdefg	0.56 ± 0.22 cd	0.52 ± 0.11 bcdef	7.85 ± 2.68 abcde
Cla cla DN987		1.40 ± 0.27 abcd	1.97 ± 0.32 defg	0.51 ± 0.17 cd	0.84 ± 0.23 ab	4.28 ± 0.76 defgh
Cla cla UT159A	LP	1.96 ± 0.65 ab	1.70 ± 0.20 defg	0.60 ± 0.24 cd	0.27 ± 0.07 efg	4.15 ± 1.19 efgh
Cla etu MG106	HP	0.54 ± 0.13 fg	3.45 ± 0.45 ab	1.24 ± 0.38 bc	0.29 ± 0.10 efg	8.36 ± 0.88 abc
Cla etu MX116A		0.59 ± 0.20 fg	2.41 ± 0.62 bcdef	0.51 ± 0.11 cd	0.39 ± 0.08 cdefg	2.77 ± 0.19 gh
Cla etu SP108C	MP	0.62 ± 0.17 efg	2.94 ± 0.73 abcd	0.93 ± 0.23 cd	0.35 ± 0.06 cdefg	7.91 ± 1.34 abcde
Fun mos CU114		0.99 ± 0.20 defg	2.34 ± 0.58 bcdefg	0.78 ± 0.26 cd	0.38 ± 0.11 cdefg	5.40 ± 0.96 bcdefg
Fun mos HO102		0.81 ± 0.09 efg	2.15 ± 0.23 cdefg	0.49 ± 0.12 cd	0.31 ± 0.03 defg	4.42 ± 1.26 cdefgh
Fun mos NB114	HP	0.58 ± 0.15 fg	3.88 ± 1.33 a	0.82 ± 0.27 cd	0.18 ± 0.07 efg	8.07 ± 2.99 abcde
Gig mar JA201A		0.59 ± 0.19 fg	1.65 ± 0.30 efg	0.60 ± 0.23 cd	0.59 ± 0.28 bcde	5.98 ± 1.07 bcdefg
Gig mar MR104		0.55 ± 0.20 fg	1.79 ± 0.12 defg	0.68 ± 0.30 cd	0.31 ± 0.05 defg	5.11 ± 1.08 bcdefg
Gig mar WV205A	MP	0.96 ± 0.29 defg	1.69 ± 0.21 defg	0.70 ± 0.22 cd	0.28 ± 0.06 efg	6.89 ± 1.35 bcdef

Fungal isolate	PL	Inorganic phosphate in mg	Lipid-phosphate in mg	Long-chained poly-P in mg	Short-chained poly-P in mg	DNA-, RNA-, protein-phosphate in mg
Par occ CR102	LP	0.91 ± 0.46 defg	1.69 ± 0.45 defg	0.41 ± 0.10 d	0.31 ± 0.05 defg	6.00 ± 1.65 bcdefg
Par occ HA771	MP	0.76 ± 0.14 efg	1.94 ± 0.21 defg	0.77 ± 0.20 cd	0.21 ± 0.06 efg	6.88 ± 1.77 bcdef
Par occ OR924	HP	0.52 ± 0.14 fg	1.93 ± 0.19 defg	0.84 ± 0.29 cd	0.44 ± 0.13 bcdefg	7.99 ± 0.83 abcde
Rhi int KE103		0.68 ± 0.10 efg	1.58 ± 0.14 efg	0.37 ± 0.18 d	0.19 ± 0.08 efg	4.26 ± 1.29 defgh
Rhi int ON.pr.Te3	LP	0.80 ± 0.11 efg	1.39 ± 0.18 fg	0.16 ± 0.06 d	0.06 ± 0.02 g	3.25 ± 0.49 fgh
Rhi int TU101		2.10 ± 0.55 a	1.40 ± 0.15 fg	0.45 ± 0.13 cd	0.86 ± 0.30 a	3.39 ± 0.43 fgh
Rhi irr QB000	LP	1.64 ± 0.26 abcd	1.78 ± 0.46 defg	0.47 ± 0.10 a	0.53 ± 0.27 bcdefg	3.84 ± 0.65 h

Table S8. Results of the statistical tests (effect on biomass and P contents and tissue concentrations) and the linear regression analysis (LRA). The LSD tests in the table describe tests in which the isolates were grouped and compared according to their performance levels. Isolates were only grouped when the tests with the individual isolates suggested that there were significant differences between the different performance levels but not within one performance level. All tests were conducted with the statistical program UNISTAT 6. Shown are here only the statistically significant results (all other results $p > 0.05$).

No.	Comparison	Test	Output	p
1	Total plant biomass: All treatments	ANOVA	$F_{31,99} = 4.785$	$p < 0.0001$
2	Total plant biomass: Isolates of *A. morrowiae* and *R. intraradices/irregulare*	ANOVA	$F_{1,26} = 4.512$	$p < 0.0433$
3	Total plant biomass: Performance groups	ANOVA	$F_{2,73} = 41.303$	$p < 0.0001$
4	Total plant biomass: High performance and controls	LSD		$p < 0.0001$
5	Total plant biomass: High performance and low performance	LSD		$p < 0.0001$
6	Total plant biomass: High performance and medium performance	LSD		$p < 0.0001$
7	Total plant biomass: Medium performance and low performance	LSD		$p = 0.0001$
8	Root biomass and root P content (LRA)	$F_{1,30} = 18.554$	$r^2 = 0.382$	$p = 0.0002$
9	Shoot biomass and shoot P content (LRA)	$F_{1,30} = 4.384$	$r^2 = 0.127$	$p = 0.045$
10	Root P content: All isolates	ANCOVA	$F_{32,97} = 1.926$	$p < 0.0077$
11	Root P content: All isolates with performance levels	ANCOVA	$F_{4,78} = 4.136$	$p = 0.0043$
12	Root P content: High performance and controls	LSD		$p = 0.019$
13	Root P content: High performance and low performance	LSD		$p = 0.0021$
14	Root biomass and root P tissue concentration (LRA)	$F_{1,30} = 5.596$	$r^2 = 0.157$	$p = 0.0247$
15	Shoot biomass and shoot P tissue concentration (LRA)	$F_{1,30} = 4.053$	$r^2 = 0.119$	$p = 0.0531$
16	Shoot P content: All treatments	ANCOVA	$F_{32,97} = 3.001$	$p < 0.0001$
17	Shoot P content: High performance and controls	LSD		$p = 0.0045$
18	Shoot P content: High performance and medium performance	LSD		$p = 0.0186$
19	Shoot P content: Low performance and controls	LSD		$p = 0.0214$
20	Shoot P tissue concentration: All treatments	ANCOVA	$F_{32,97} = 2.725$	$p < 0.0001$
21	Shoot P tissue concentration: Low performance and controls	LSD		$p = 0.0039$
22	Shoot P tissue concentration: Low performance and medium performance	LSD		$p = 0.0001$
23	Shoot P tissue concentration: Low performance and high performance	LSD		$p < 0.0001$

Table S9. Results of the statistical tests (N contents and N tissue concentrations) and the linear regression analysis (LRA). The LSD tests in the table describe tests in which the isolates were grouped and compared according to their performance levels. Isolates were only grouped when the tests with the individual isolates suggested that there were significant differences between the different performance levels but not within one performance level. All tests were conducted with the statistical program UNISTAT 6. Shown are here only the statistically significant results (all other results $p > 0.05$).

No.	Comparison	Test	Output	p
1	Root biomass and root N content (LRA)	$F_{1,30} = 563.3$	$r^2 = 0.9494$	$p < 0.0001$
2	Root biomass and root N tissue concentration (LRA)	$F_{1,30} = 114.0$	$r^2 = 0.7917$	$p < 0.0001$
3	Shoot biomass and shoot N content (LRA)	$F_{1,30} = 584.7$	$r^2 = 0.9511$	$p < 0.0001$
4	Shoot biomass and N tissue concentration (LRA)	$F_{1,30} = 108.5$	$r^2 = 0.7833$	$p < 0.0001$
5	Root N content: All treatments	ANCOVA	$F_{32,97} = 35.936$	$p < 0.0001$
6	Root N content: All isolates with performance levels	ANCOVA	$F_{4,78} = 161.688$	$p < 0.0001$
7	Root N content: High performance and controls	LSD		$p < 0.0001$
8	Root N content: High performance and medium performance	LSD		$p < 0.0001$
9	Root N content: High performance and low performance	LSD		$p < 0.0001$
10	Root N content: Medium performance and controls	LSD		$p = 0.0045$
11	Root N content: Medium performance and low performance	LSD		$p = 0.0003$
12	Root N tissue concentration: All treatments	ANCOVA	$F_{32,97} = 7.957$	$p < 0.0001$
13	Root N concentration: All isolates with performance levels	ANCOVA	$F_{4,78} = 32.846$	$p < 0.0001$
14	Root N concentration: High performance and controls	LSD		$p < 0.0001$
15	Root N concentration: High performance and medium performance	LSD		$p < 0.0001$
16	Root N concentration: High performance and low performance	LSD		$p < 0.0001$
17	Root N concentration: Medium performance and controls	LSD		$p = 0.0027$
18	Root N concentration: Medium performance and low performance	LSD		$p = 0.002$
19	Shoot N content: All treatments	ANCOVA	$F_{32,97} = 27.792$	$p < 0.0001$
20	Shoot N content: All isolates with performance levels	ANCOVA	$F_{32,97} = 174.317$	$p < 0.0001$
21	Shoot N content: High performance and controls	LSD		$p < 0.0001$
22	Shoot N content: High performance and medium performance	LSD		$p < 0.0001$
23	Shoot N content: High performance and low performance	LSD		$p < 0.0001$
24	Shoot N content: Medium performance and controls	LSD		$p = 0.012$

25	Shoot N content: Medium performance and low performance	LSD		p = 0.0004

26	Shoot N tissue concentration: All treatments	ANCOVA	$F_{32,97} = 6.825$	$p < 0.0001$
27	Shoot N concentration: All isolates with performance levels	ANCOVA	$F_{4,78} = 33.227$	$p < 0.0001$
28	Shoot N concentration: High performance and controls	LSD		$p < 0.0001$
29	Shoot N concentration: High performance and medium performance	LSD		$p < 0.0001$
30	Shoot N concentration: High performance and low performance	LSD		$p < 0.0001$
31	Shoot N concentration: Medium performance and controls	LSD		p = 0.010
32	Shoot N concentration: Medium performance and low performance	LSD		p = 0.0007

Table S10. Results of the linear regression analysis (LRA) between colonization of the plants with mycorrhizal fungi or with root nodules and plant growth or nutrient parameters. All tests were conducted with the statistical program UNISTAT 6.

No.	Comparison	Test	Output	p
1	Total arbuscular volume and total plant biomass (LRA)	$F_{1,29} = 5.32$	$r^2 = 0.1551$	$p = 0.0283$
2	Root nodulation (d. wt.) and shoot N content (LRA)	$F_{1,30} = 0.025$	$r^2 = -0.0324$	$p = 0.8754$
3	Root nodulation (d. wt.) and shoot N concentration (LRA)	$F_{1,30} = 0.198$	$r^2 = -0.0266$	$p = 0.6599$
4	Root nodulation (d. wt.) and root N concentration (LRA)	$F_{1,30} = 0.065$	$r^2 = -0.0311$	$p = 0.7999$
5	Root nodulation (d. wt.) and root N content (LRA)	$F_{1,30} = 0.314$	$r^2 = -0.0226$	$p = 0.5792$
6	Root nodulation (d. wt.) and root biomass (LRA)	$F_{1,30} = 0.285$	$r^2 = -0.0236$	$p = 0.5977$
7	Root nodulation and shoot biomass (LRA)	$F_{1,30} = 0.001$	$r^2 = -0.0332$	$p = 0.9731$
8	Root nodulation and total biomass (LRA)	$F_{1,30} = 0.726$	$r^2 = -0.0291$	$p = 0.7264$

Table S11. Results of the statistical tests (P pool distribution). The LSD tests in the table describe tests in which the isolates were grouped and compared according to their performance levels. Isolates were only grouped when the tests with the individual isolates suggested that there were significant differences between the different performance levels but not within one performance level. All tests were conducted with the statistical program UNISTAT 6. Shown are here only the statistically significant results (all other results $p > 0.05$).

No.	Comparison	Test	Output	p
1	Correlation between root biomass and LP	Pearson	$r_{(30)} = 0.7303$	$p < 0.0001$
2	Correlation between root biomass and DNA-P	Pearson	$r_{(30)} = 0.8570$	$p < 0.0001$
3	Percentage P_i allocation: All isolates	ANCOVA	$F_{32,97} = 2.830$	$p < 0.0001$
4	Percentage P_i: Low performance and high performance	LSD		$p < 0.0001$
5	Percentage P_i: Low performance and medium performance	LSD		$p = 0.0001$
6	Percentage P_i: Low performance and controls	LSD		$p = 0.026$
7	Correlation root biomass and P_i tissue concentration	Pearson	$r_{(32)} = - 0.4401$	$p = 0.0117$
8	P_i tissue concentration: All treatments	ANCOVA	$F_{32,97} = 4.304$	$p < 0.0001$
9	P_i tissue concentration: Low performance and high performance	LSD		$p < 0.0001$
10	P_i tissue concentration: Low performance and medium performance	LSD		$p < 0.0001$
11	P_i tissue concentration: Low performance and controls	LSD		$p = 0.014$
12	P_i content: All treatments	ANCOVA	$F_{32,97} = 2.544$	$p = 0.0002$
13	P_i content: Low performance and medium performance	LSD		$p = 0.036$
14	P_i content: Low performance and controls	LSD		$p = 0.023$
15	Long-chained poly-P content: All treatments	ANCOVA	$F_{32,97} = 6.777$	$p < 0.0001$
16	Long-chained poly-P content: High performance and controls	LSD		$p = 0.0037$
17	Long-chained poly-P content: High performance and low performance	LSD		$p = 0.0002$
18	Long-chained poly-P content: High performance and medium performance	LSD		$p = 0.019$
19	Correlation root biomass and short-chained poly-P tissue concentration	Pearson	$r_{(32)} = - 0.543$	$p = 0.0013$
20	Short-chained tissue concentration: All treatments	ANCOVA	$F_{32,97} = 2.866$	$p < 0.0001$
21	Short-chained poly-P tissue concentration: Low performance and controls	LSD		$p = 0.018$
22	Short-chained poly-P tissue concentration: Low performance and medium performance	LSD		$p = 0.008$
23	Short-chained poly-P tissue concentration: Low performance and high performance	LSD		$p = 0.0001$
24	Correlation root biomass and long-chained to short-chained poly–P ratio	Pearson	$r_{(30)} = 0.6950$	$p < 0.0001$

CHAPTER 6: COMMON MYCORRHIZAL NETWORKS AND THEIR EFFECT ON THE BARGAINING POWER OF THE FUNGAL PARTNER IN THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS

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6.1 ABSTRACT

Arbuscular mycorrhizal (AM) fungi form mutualistic interactions with the majority of land plants, including some of the most important crop species. The fungus takes up nutrients from the soil, and transfers these nutrients to the mycorrhizal interface in the root, where these nutrients are exchanged against carbon from the host. AM fungi form extensive hyphal networks in the soil and connect with their network multiple host plants. These common mycorrhizal networks (CMNs) play a critical role in the long-distance transport of nutrients through soil ecosystems and allow the exchange of signals between the interconnected plants. CMNs affect the survival, fitness, and competitiveness of the fungal and plant species that interact via these networks, but how the resource transport within these CMNs is controlled is largely unknown. We discuss the significance of CMNs for changes in plant communities and for the bargaining power of the fungal partner in the AM symbiosis.

6.2 INTRODUCTION

The arbuscular mycorrhizal symbiosis between plants and fungi is formed by approximately 65% of all known land plant species and many plants depend on this symbiosis for their nutrient supply.(Wang & Qiu, 2006) Many fungi also provide non-nutritional benefits to their host that are critical for plant survival or fitness, including protection against pathogens, or improved resistance against drought and salinity.(Smith & Read, 2008) AM interactions are therefore essential components of large-scale

ecosystem processes and act as 'ecosystem engineers' of plant communities.(Cameron, 2010)

All AM fungi belong to the phylum Glomeromycota, and are unable to complete their life cycle without the carbon supply from their host.(Redecker & Raab, 2006) The obligate biotrophy of AM fungi and the observation that plants often suppress the AM colonization of their root system when nutrients are readily available, has led to the overall assumption that the host plant is in control of the symbiosis.(Smith & Smith, 2012) However, this phyto-centric view disregards the long co-evolution of both partners in the AM symbiosis (~ 450 Million years) that allowed the fungus to develop strategies to improve its bargaining power despite its obligate biotrophic life cycle.(Kiers *et al.*, 2011; Fellbaum *et al.*, 2012; Fellbaum *et al.*, 2014)

6.2 RESULTS

As illustrated in **Figure 1**, AM fungi and their plant partners form a complex network of many-to-many interactions, in which a single plant host is colonized by multiple fungal species, and fungal 'individuals' interact with multiple plant hosts and species simultaneously and interconnect plants by a common mycorrhizal network (CMN). Both partners in the symbiosis can choose among multiple trading partners and do not depend on a single partner for their carbon or nutrient resources. CMNs can connect plants of the same or of different plant species and of different developmental stages, and are involved in the long distance transport of nutrients (carbon, phosphate, nitrogen, or micronutrients), water, stress chemicals, and allelochemicals in soil ecosystems.(Voets *et*

al., 2008; Barto *et al.*, 2011; Babikova *et al.*, 2013; Babikova *et al.*, 2013; Weremijewicz & Janos, 2013; Gorzelak *et al.*, 2015; Teste *et al.*, 2015) Multiple fungal and plant species interact and 'communicate' via these CMNs and there is growing evidence that CMNs affect the survival and fitness, behavior and competitiveness of the plants and fungi that are linked via these networks.

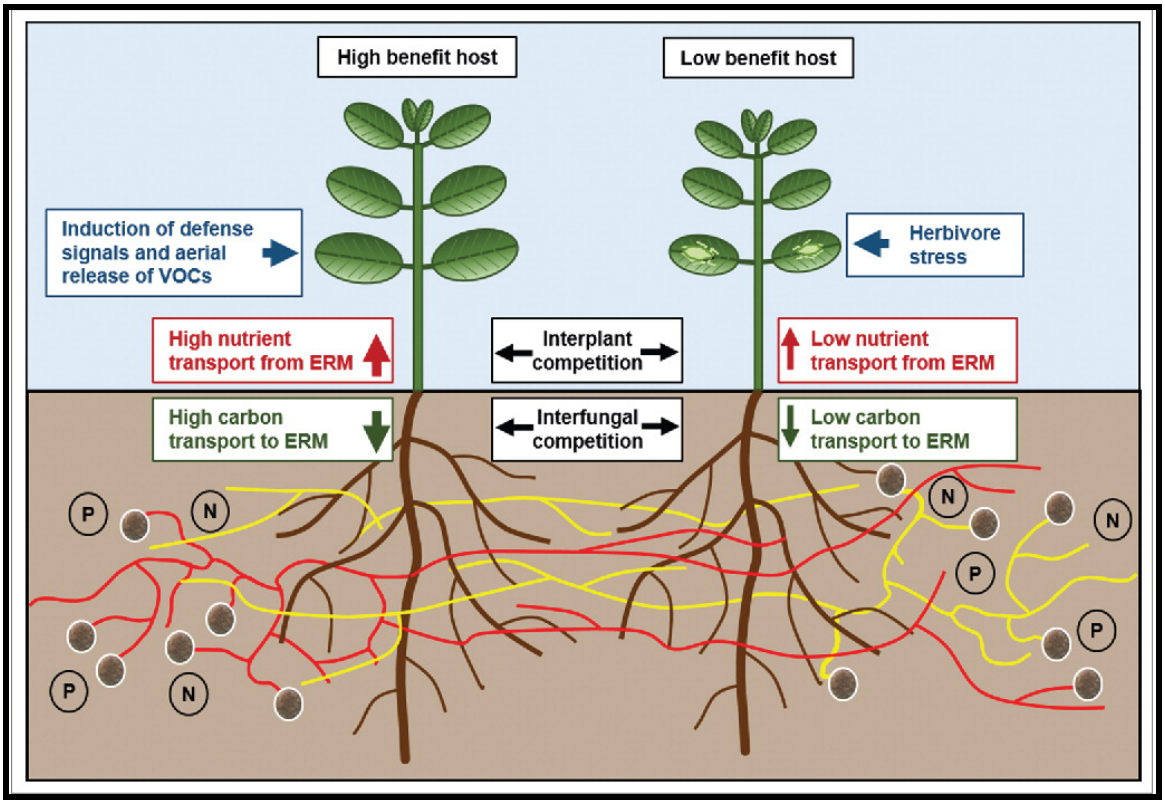

Figure 1. Function of common mycorrhizal networks (CMNs) in soil ecosystems. The roots of plants are connected by CMNs of single or multiple arbuscular mycorrhizal (AM) fungal morphospecies. Plants compete with their carbon resources for nutrients that become available for their CMNs. Plants can differ in their carbon transport to the CMNs and can represent low or high benefit hosts for the AM fungus. Low benefit host plants within a CMN could be for example seedlings that compete with adult plants, or adult plants that transfer less carbon to the CMN due to shading or herbivore damage. AM fungi can discriminate between low and high quality host plants and preferentially transfer resources to high quality hosts what can contribute to the inequalities among plants that have been observed in studies with CMNs. In addition, CMNs can serve as a conduit for the transfer of warning signals or of allelochemicals between plants within one CMN. Warning signals that are formed by donor plants for example in response to herbivore stress can lead in receiver plants to an induction of defense reactions and the release of volatile organic compounds from the leaves (VOCs). Directed transport of allelochemicals to specific plants via CMNs can facilitate the interplant competition and suppress the growth of plant competitors. Fungal CMNs compete for soil nutrients and compete with these nutrients for carbon resources from the different host plants within their CMN.

The development of CMNs allows the fungus to gain access to multiple trading partners, and ensures a continuous carbon supply for the fungus even when one host plant loses its ability to transfer resources to the fungal partner by e.g. pathogen or herbivore damage or by early senescence. When AM fungi are able to discriminate between host plants within their CMN, the fungus gains bargaining power because the plants within its network are forced to compete. In theory, natural selection should favor those fungi that are able to establish a CMN with many host plants, because inter-plant competition will force the competing plants to transfer more carbon to their fungal partner in order to receive a greater share of nutrients from the CMN (Wyatt *et al.*, 2014).

In order to better understand how nutrient transport among plants in CMNs is controlled, we examined the fungal phosphate and nitrogen allocation to plants that differed in their ability to provide carbon to their fungal symbiont (low and high quality hosts). The studies demonstrated that fungi were indeed able to discriminate among plants that shared a CMN and preferentially allocated nutrient resources to host plants that were able to provide more carbon benefit.(Fellbaum *et al.*, 2014) Nutrient allocation within the CMN, however, was not controlled on an all-or-none basis, and the fungus also transferred phosphate and nitrogen to low quality hosts, and maintained a high colonization rate in these plants. Host plant quality does not seem to be an important factor for root colonization,[7] and AM fungi also invest resources to actively colonize the roots of low quality hosts.(Knegt *et al.*, 2014) The strategy to colonize both, low and high quality host plants ensures that the loss of a high quality host is less detrimental for the fungus, and forces also high quality hosts to compete for nutrients from the CMN.

Both partners in the AM symbiosis are able to discriminate between different symbiotic partners, and it has been suggested that the 'fair trade' between both partners contributed to the evolutionary stability of the AM mutualism.(Kiers *et al.*, 2011) Carbon to nutrient exchange ratios at the mycorrhizal interface are controlled by resource supply and demand and follow biological market dynamics.(Kiers *et al.*, 2011; Fellbaum *et al.*, 2012; Fellbaum *et al.*, 2014) Consistently, we found that in the absence of choice, the fungus transfers more nutrient resources per unit carbon to low quality hosts.(Fellbaum *et al.*, 2014) When the fungus has only access to low quality hosts, the dependency of the fungus for host plant´s carbon shifts the cost to benefit ratio at the mycorrhizal interface in favor of the host.

When plants invest carbon resources into a fungal network that also benefits their competitors, the preferential nutrient allocation to specific host plants within a CMN will provide the favored host plants with a net benefit to the detriment of the unfavored plants within the CMN.(Selosse *et al.*, 2006) Plant species or individuals of one species can differ in their carbon investment into the CMN,(Walder *et al.*, 2012) and CMNs have been shown to amplify inequalities in plant communities,(Booth & Hoeksema, 2010; Weremijewicz & Janos, 2013) and between seedlings and established adult plants that are connected by a CMN. While some studies have shown that seedlings can benefit from established CMNs with adult plants,(van der Heijden & Horton, 2009) other studies demonstrated negative impacts of CMNs on seedling establishment and fitness, and P nutrition.(Kytöviita *et al.*, 2003; Pietikäinen & Kytöviita, 2007; Merrild *et al.*, 2013) When AM fungi are able to discriminate among plants within their CMN, the fungal

partner should provide more resources to adult plants due to their higher carbon transport to the CMN.

The suppression of plants within CMNs, however, can also be a plant-mediated effect. Allelochemicals, root-secreted secondary metabolites that plants use to regulate the rhizosphere to the detriment of competing neighboring plants have also been shown to be transferred from donor to target plants by CMNs.(Barto *et al.*, 2011; Barto *et al.*, 2012) It is currently unknown, whether AM fungi are able to control the transfer of allelochemicals within their CMNs, but it is interesting to speculate that AM fungi by a directed transport of allelochemicals could suppress specific plants within their CMN, or susceptible fungal competitors. Some plants release allelochemicals with antifungal activities, and it has been shown that some invasive plants use these antifungal allelochemicals to suppress the mycorrhizal colonization of their native plant competitors.(Stinson *et al.*, 2006)

CMNs play also an important role in the plant-to-plant 'communication' and transfer infochemicals and warning signals between plants. Plants that are attacked by herbivores produce volatile organic compounds that act as a repellent for aphids but attract the natural enemies of aphids to the infested leaves. These volatiles are only produced by non-infested plants when they share a CMN with infested plants.(Babikova *et al.*, 2013) These warning signals between plants within one CMN are transmitted very rapidly, and non-infested plants up-regulated genes of the jasmonate defense pathway shortly after plants within their CMN were attacked by herbivores.(Song *et al.*, 2014) Herbivore damage can reduce the capability of plants to provide the CMN with carbon, and AM fungi that efficiently share these defense-related signals with other plants within their

CMN will be able to reduce the negative impact of herbivore damage on their carbon supply. It is currently not known whether the fungus controls the flow of these defense-related signals within its CMN. The fungus could transfer these warning signals preferentially to host plants that provide more carbon benefit, or to host plants that demonstrate the strongest defense response in order to keep the damage to these plants as small as possible(Babikova *et al.*, 2013). Or the fungus could share these warning signals equally among the plants within its CMN, because the fungus is unable to predict how severely the carbon flow of individual plants will be affected by herbivore damage. Some plants respond to a herbivore attack above-ground with an increased carbon allocation below ground into roots and root exudates. This could increase the carbon transport of these plants into the CMN, and could improve the attractiveness of these plants for fungal colonization and signal transduction.(Holland *et al.*, 1996)

6.3 CONCLUSIONS

AM fungi and their CMNs play a significant role in plant ecosystems and control the fitness and competitiveness of the plant individuals within their CMNs. Our current understanding about resource exchange in the AM symbiosis is primarily based on experiments with root organ cultures or with single plants that are colonized by one AM fungus.(Kiers *et al.*, 2011; Fellbaum *et al.*, 2012) The transferability of these experiments to CMNs, however, is very limited, because in natural ecosystems both partners in the AM symbiosis can choose among multiple trading partners and do not depend on a single partner for their nutrient or carbon supply. Plants play a critical role for the carbon supply

of their CMNs and also the composition of the plant community within one CMN has been shown to affect the abundance or extension of CMNs in soils.(Derelle *et al.*, 2012; Engelmoer & Kiers, 2015) Very little is known about how AM fungi allocate nutrient resources or infochemicals within their CMN, or how host plants compete with other plants for nutrients that are available for their CMNs. More research is needed to better understand how the costs and benefits of the AM symbiosis are controlled in CMNs, and how fungal networks affect the inter-fungal or inter-plant competitiveness of both partners in natural ecosystems.

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CHAPTER 7

7.1. DISCUSSION

The application of AM fungi in a sustainable agriculture is still hindered by the lack of knowledge about their metabolic pathways and their regulation. Research has clearly demonstrated that the AM symbiosis plays an important key role in the nutrient exchange between a fungal symbiont and its plant host (Bago *et al.*, 2003; Read & Perez- Moreno, 2003; Smith & Smith, 2011), soil communities and their ecological environments (Smith & Smith, 2011; Hodge & Storer, 2015). Over evolutionary time, one would expect the selection of plants and AM fungi that cheat to increase their reproductive success at the expense of other partners and this would destabilize the AM symbiosis, but this mutualistic association still persists for more than 450 million years. However, several significant questions were still unanswered as to what triggers nutrient exchange and the physiological mechanisms employed by partners to control the interactions in AM symbiosis. To answer these, we therefore hypothesized the following: 1. Host plants C has an effect on AM fungal P uptake and the transport to the host plant, 2. Host plants and AM fungi can discriminate between beneficial and less beneficial partners and in return, reward these partners accordingly and 3. Plant growth benefit is affected by fungal P and N metabolism.

To better understand the discrimination between partners in the AM symbiosis, we used *in vitro* root organ cultures and whole plant systems and selected AM fungal species that differed in their behavior and their cooperative growth benefits (Kiers *et al.*, 2011).

We treated these systems with ^{33}P and ^{14}C to track the exchange of resources between partners (Kiers *et al.*, 2011). We found that the AM fungi exhibited high and low levels of cooperation based on the costs of carbon per unit phosphate (P) and nitrogen (N) transferred and their resource hoarding strategies. The results demonstrated that AM fungi are able to distinguish roots that differ in the benefit that they provide and allocate P accordingly to roots that are able to provide more carbon (C). We were able to also demonstrate that an increasing amount of C transferred by the host stimulated P transport to the root by the cooperative fungus but did not affect the transport of the less-cooperative fungus. Our results provided strong evidence that AM fungi play an important role in regulating nutrient exchange and the different host plant competition between multiple hosts available can shift nutrients to carbon exchange in the AM symbiosis to the advantage of the fungus contribution (Kiers *et al.*, 2011; Fellbaum *et al.*, 2012; Fellbaum *et al.*, 2014; Mensah *et al.*, 2015).

Overall, our work strongly suggests that AM fungi are able to discriminate between host plant partners. This confirms the results of Bever *et al.* (2009), who found that host plants are able to discriminate between more beneficial and less beneficial fungal species in a segregated split root system. Our results validated evidence that indeed reciprocal of C for P and N exchange mechanisms do exist in the AM symbiosis (Bücking & Shachar- Hill, 2005; Hammer *et al.*, 2011; Fellbaum *et al.*, 2012) and high intraspecific variation within the morphospecies contributes to the high phenotypic and functional diversity (Koch *et al.*, 2006).

To address how cooperative behavior between symbionts is enforced, we conducted a study in a whole plant system (*M.truncatula*) and manipulated cooperative behavior by

supplying different nutrient conditions and tracked the exchange of resources between partners. The carbon (C) flux through the plant to the fungal partners was studied by stable isotope probing (SIP) and we found that more carbon was integrated into the RNA of the more cooperative fungus (*Rhizophagus intraradices*) compared to the less cooperative fungi (*G. custos & G.aggregatum)*(Kiers *et al.,* 2011). However, the ability of the AM fungi to provide resource benefit is dependent on what benefits are available to that particular fungus than to other fungi (Werner *et al.*, 2014). This reciprocal reward system is analogous to a market economy where trade is favored with partners offering the best rate of exchange.

AM interactions are however, one of the most complex associations to understand because plant and their fungal symbionts interact in complex networks with multiple partners, which should select against cooperation and reduce the effectiveness of mechanisms that could enforce cooperation behavior. To test this hypothesis, we used two *Medicago truncatula* plants inoculated with two AM fungal species (*G.aggregatum* or *R. irregularis*) in whole plant systems. ^{15}N and ^{33}P were applied and tracked fungal nutrient transport in a common mycorrhizal network (Fellbaum *et al.*, 2014).The plants were shaded to control the photosynthetic activities either by covering one or both plants. Our results strongly indicate that both AM fungi preferentially allocate resources to the unshaded host plant (Fellbaum *et al.*, 2014). Also, AM fungi transferred more P and N to shaded host plants when the AM fungus had no choice between high and low quality host plants (Fellbaum *et al.*, 2014). Interestingly, AM fungi were able to maintain high levels of colonization in systems with one unshaded and the other shaded host plants. This suggests that the fungus used its C resource from the unshaded plant to maintain the level

of colonization in the shaded plant, suggesting that one fungal strategy is to always have access to a C source even if the host plant is a low quality host (Fellbaum *et al.*, 2014).

Plant growth responses following colonization with different isolates of a single species of AM fungus can range from highly beneficial to detrimental, but the reasons for this high within-species diversity are currently unknown. We found in our previous study that colonization by less-cooperative fungus resulted in a higher C cost per unit P transferred to the host plant compared to the more-cooperative fungus (Kiers *et al.*, 2011). It is predicted that evolutionary theory of sanctions on plants would decrease the less cooperative fungus's reproductive success and would eventually reduce fungal diversity. So to examine whether differences in growth and nutritional benefits are related to the P and N metabolism of the fungal symbiont, we studied the effect of 31 different isolates from 10 AM fungal morphospecies on the P and N nutrition of *Medicago sativa* and the P allocation among different P pools. Our results demonstrate that there is a high within fungal species diversity in the efficiency with which AM fungi contribute to the N nutrition of the host plant (Mensah *et al.*, 2015) but the reasons for this high within-species diversity are currently unknown. There are indications that differences in the fungal polyP metabolism could play a role in this diversity (Mensah *et al.*, 2015), but it has also been suggested that the nutrient transport efficiency could mainly be the result of the compatibility between a fungal symbiont and its plant host. The results in our previous studies validate that the less-cooperative fungus withheld P as inaccessible long-chain polyp showing the difference in fungal cooperation and their hoarding strategies (Kiers *et al.*, 2011). These results provide strong evidence that the long-chain poly-P

pool stores P in the AM fungal hyphae whereas short-chain poly-P are good indicators of P transport to the host plant (Takanishi *et al.*, 2009; Kiers *et al.*, 2011).

Overall, our studies support the hypotheses that the fungal P and N transport are affected by the C supply of the host plant and the biological market theory provided evidence that AM fungi and host plant discriminate between partners through their many to many nutrient exchange interactions in the AM symbiosis (Werner *et al.,* 2014).

7.2. FUTURE EXPERIMENTS

The results from our previous studies have demonstrated that resource exchange between host plants and AM symbionts are driven by biological market dynamics where both partners are able to identity better partners that are able to provide more benefit, and reciprocally reward resources (in terms of N and P for C) to the partners offering the best exchange rate (Kiers *et al.*, 2011; Fellbaum *et al.*, 2012; Fellbaum *et al.*, 2014). However, there are still questions that need to be answered since these studies only focused on a fraction of the total benefits in AM symbiosis. Our results showed also that there is high within fungal species diversity in the nutrient benefits to the host plants (Mensah *et al.*, 2015). However, further studies should focus on the role of the fungal polyP metabolism and nitrogen in nutrient uptake and transport efficiency of the fungal partners. It is therefore important for the plant to maintain high multiple AM inter-fungal competitions where environmental conditions can affect the changes to nutrient exchange in AM community. The AM fungus beneficial status may be dependent on what benefits or resources that particular fungus has in possession to compare with other AM fungi

(Werner *et al.*, 2014). The host plant and AM fungi form multiple interactions in the AM symbiosis. However, the AM fungi can differ in the nutritional benefits they provide to the host plant through CMN (Fellbaum *et al.*, 2014; Mensah *et al.*, 2015). The benefit in the AM symbiosis for the host plant is the sum of the benefits that are provided by all the AM fungi interacting with an individual host plant. However, little is known about how the composition of these communities is controlled. Our study focus on how nutrients depend on the host plant and how the availability of nutrients for AM fungi plays an important driving force that shape the AM fungal community composition. We used *Medicago truncatula* as our model plant – (non-mycorrhizal target and mycorrhizal donor plants) where the plants were inoculated with either *Glomus aggregatum* (GA) or *Rhizophagus irregularis* (RI). The plants were grown under low nutrient supply conditions in their respective T-shaped PVC pipe compartments, before the compartments were connected with 6-cm-long PVC pipes and 50-µm nylon mesh forming three connected compartments (GA-Target-RI). This allowed the fungi to crossover the membrane from the donor to the target plant compartment. After two weeks of varying the amount of P and N supply for the donor and target plants, the plants were harvested, analyzed for biomass, ^{15}N, P contents and their mycorrhizal colonization by microscopy and qPCR. Our initial data (Mensah *et al*. in prep.) indicate that the community composition depended on both the nutrient demand of the target plant and the access of nutrients for individual fungal species. This supports the view that plants have unique AM fungal communities that they are associated with (Hausmann & Hawkes, 2009). Our preliminary results also clearly indicated that the host plant plays an important role in the AM fungal community composition (Pendergast *et al.*, 2013; Zobel & Öpik,

2014). The results also showed that the less – cooperative fungi (GA) were able to outcompete the more-cooperative fungi (RI) when they have access to the specific nutrient the host plant is in demand. However, the more – cooperative fungi (RI) was dominant under both low and high nutrient supply conditions unlike the less-cooperative fungi (GA) which was only dominant when it had access to the specific nutrients the host plant was in demand of (Mensah *et al.* in prep.). This study supports the hypothesis that the reciprocal reward of specific nutrients act as an important driving force that control the shape of AM communities in the AM symbiosis that is controlled by biological market dynamic.

Even though these studies would not answer all the questions that pertain in the AM symbiosis, we hope that these studies go in the right direction to further advance our understanding of cooperation in the AM symbiosis and their importance in sustainable agriculture.

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