

Evolution of the cooperation and consequences of a decrease in plant diversity on the root symbiont diversity

Marie Duhamel

▶ **To cite this version:**

Marie Duhamel. Evolution of the cooperation and consequences of a decrease in plant diversity on the root symbiont diversity. Agricultural sciences. Université de Rennes, 2013. English. NNT : 2013REN1S045 . tel-00867233

HAL Id: tel-00867233

https://theses.hal.science/tel-00867233

Submitted on 27 Sep 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

ANNÉE 2013

THÈSE / UNIVERSITÉ DE RENNES 1
sous le sceau de l'Université Européenne de Bretagne

pour le grade de

DOCTEUR DE L'UNIVERSITÉ DE RENNES 1

Mention : Biologie

Ecole doctorale Vie-Agro-Santé

présentée par

Marie Duhamel

Préparée à l'unité de recherche UMR 6553 ECOBIO
«Ecosystèmes, Biodiversité, Evolution»
UFR SVE Science de la vie et de l'environnement

Evolution de la coopération et conséquences d'une baisse de diversité des plantes sur la diversité des symbiontes racinaires.

Evolution of the cooperation and consequences of a decrease in plant diversity on the root symbiont diversity.

Thèse soutenue à Rennes
le 24 Juin 2013

devant le jury composé de :

Matthias Rillig
Professeur - Freie Universität Berlin / *rapporteur*

Marc André Selosse
Professeur - Université de Montpellier II / *rapporteur*

Nancy Johnson
Professeur - Northern Arizona University / *examinateur*

Nico van Straalen
Professeur - Free University of Amsterdam / *examinateur*

Philippe Vandenkoornhuyse
Professeur - Université de Rennes1/ *directeur de thèse*

Toby Kiers
PhD - Free University of Amsterdam / *co-directeur de thèse*

Table of contents:

Samenvatting 7
Summary 9
Résumé 10

General introduction 13
I. Relationships among living organisms : 13
1. Symbiosis: definition 13
2. Theory: Evolutionary forces and the symbiosis dilemma 15
3. Key mechanisms contributing to stability of 'mutualistic' symbiosis 15
a. Partner choice 16
b. Vertical transmission 16
c. Obligate dependancy 16
II. Symbiosis between plants and fungi 17
1. The Arbuscular Mycorrhizal symbiosis 17
a. History, taxonomy 17
b. Toward a molecular diagnosis of fungal diversity ? 20
c. Description, characteristics of arbuscular mycorrhiza 21
d. Nutrient exchanges 24
2. No host specificity but host preference 25
3. Importance of AM symbiosis in ecosystems 26
a. Importance for plant development and reproduction 26
b. Impact of AM fungi on soil structure 27
c. Fungal and plant community structure 27
d. Link between AM symbiosis and ecosystem stability and productivity 28
III. Objectives and approaches 30

Chapter I: Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis 33
Supporting material 42
Material and Methods 42

Supplementary figures 56
Supplementary tables 65

Chapter II: Do fungivores trigger the transfer of protective metabolites from host plants to arbuscular mycorrhizal hyphae? 69
Introduction 70
Methods 72
Results 76
Discussion 81
Conclusion 84
Supplemental material 86

Chapter III: PHYMYCO-DB: A curated database for analyses of fungal diversity and evolution 97
Introduction 99
Pollution of public sequence database and the aim of PHYMYCO-DB 101
Database Structure : Design and Implementation 102
Tools within PHYMYCO-DB 106
PHYMYCO-DB as a Tool for Phylogenetic Identifications and Inferences 106
Discussion 109
Availability and Future Directions 111
Supplementing information 112

Chapter IV: Does a decrease in plant diversity lead to a decrease in AM symbiont diversity? 115
Introduction 115
Material and methods 117
Preliminary results and discussion 126
Supplementary material 137

Chapter V: Global sustainable agriculture : new possible trajectories from mutualistic symbiosis 141
Introduction 142
Intensive vs. extensive agriculture? 142
Crop selection from traits? 143
Arbuscular mycorrhiza and consequences of agriculture 144
Mutualistic strategy and agriculture 145
Future of agricultural trajectories 147
Conclusion 148

General discussion and perspectives 151
I. Exploring the bilateral control of AM symbiosis through preferential C allocation and P hoarding strategies 151
1. Functioning of AM symbiosis in a more complex experimental design 152
2. Breeding and the ability of plants to discriminate among fungal partners 152
3. Plasticity of the AM symbiosis 153
4. AM symbiosis costs and related hypotheses 153
II. Physiological and molecular bases of the sanction trait by plants 154
III. Host plant involvement in AM symbiont defense 155
IV. Plant diversity versus fungal symbiont diversity 156
1. Molecular phylogeny and taxonomy 156
2. Different levels of diversity to consider 157
3. Integrating other organisms 159
V. Arbuscular mycorrhizal symbiosis for a sustainable agriculture 160

Conclusion 162
References 163
Acknowledgments 185

List of abbreviations:

AIC: Akaike information criterion

ANOVA: analysis of variance

AM fungi: Arbuscular Mycorrhizal fungi

BLAST: Basic Local Alignment Search Tool

bp: base pair

C: carbon

13C: isotope 13 of the carbon

14C: isotope 14 of the carbon

13CO2: carbon dioxyde containing the isotope 13 of the carbon

ΔG: free energies

EF1α: elongation factor 1

EmpPCR: polymerase chain reaction in emulsion

ERM: extra radical mycelium

FCA: Factorial correspondence analysis

GLM: general linear model

ITS: internal transcribed spacer

LSU: large subunit

LTER: long term ecological research

MID: multiplex identifier

mt LSU rRNA: mitochondrial large subunit of the ribosomal RNA gene

nt: nucleotide

N: nitrogen

NGS: next generation sequencing

NMDS: non metric multidimensional scaling

OTU: operational taxonomy unit

P: phorphorus

32P: isotope 32 of the phorphorus

33P: isotope 33 of the phorphorus

PCR: polymerase chain reaction

qPCR: quantitative PCR

rDNA: ribosomal DNA

RNAseq: Whole Transcriptome Shotgun Sequencing

rRNA: ribosomal RNA

SIP: stable isotope probing

SSU: small subunit

List of the figures:

Figure 1: Fungal phylogenetic tree showing the relative position of the Glomeromycota phylum 19
Figure 2: Phylogenetic tree of the Glomeromycota phylum 20
Figure 3: Different structures of arbuscular mycorrhizal fungi 22
Figure 4: Autofluorescence micrograph of arbuscular mycorrhizal fungi 23
Figure 5: The different steps of root colonization 24
Figure 6: Pair-wise comparisons of carbon allocation patterns 35
Figure 7: Triple-plate experiments to mimic partner cooperation or defection 38
Figure 8: Simultaneous measurement of P and C exchange 40
Figure 9: Effects of AM fungal species *G. intraradices* and the fungivore *F. candida* on *P. lanceolata* aboveground and belowground biomass 77
Figure 10: Effect of *F.candida* presence and absence on the mean dry weight of *G. intraradices* hyphae, mtLSU copies of *G. intraradices* per gram of soil, mtLSU copies per gram of root 78
Figure 11: Effects of AM fungal species *G. intraradices* and the fungivore *F. candida* on verbascoside, aucubin, and catalpol concentration in *P. lanceolata* shoots and roots 79
Figure 12: Catalpol concentration in hyphae of *G. intraradices* and in mixed treatment 80
Figure 13: Flowchart of the data in the PHYMYCO-DB 103
Figure 14: Visualisation of sequences deleted by the manual curation after alignment 105
Figure 15: SSU rRNA phylogenetic positions of deep-sea Chytridiomycota along with the closest known related SSU rRNA fungal sequences 108
Figure 16: Cedar Creek biodiversity experiment, Univ Minnesota, USA 117
Figure 17: Steps of the root sampling 120
Figure 18: Principle of the 454 GS FLX sequencing 123
Figure 19: Details of the amplicon-sequence analysis pipeline 124
Figure 20: Structuration of the AM fungal community in function of the resemblance in OTUs between the roots from the different soil cores 127
Figure 21: Phylogenetic analysis of the SSU rRNA AM fungal OTU representative sequences 129
Figure 22: Comparisons among AM fungal communities: OTU richness per root sample and FCA results 130
Figure 23: Relative occurrence (presence/absence) of each of the 54 OTUs for the different modalities 131
Figure 24: Concordance index analyses 133
Figure 25: Occurence of the 3 clusters: group1, group2 and group3, depanding of the plant spescies richness 134
Figure S1: Schematic drawing of AM mutualism and resource exchange processes 56

Figure S2: The detection of plant-derived C fluxes into microbial nucleic acids by SIP 57
Figure S3: Growth benefits conferred by the three AM fungal species and non-mycorrhizal controls 58
Figure S4: Mycorrhizal growth characteristics of the three AM fungal species 59
Figure S5: Changes in the abundance of different AM fungal species in association with *M. Truncatula* 60
Figure S6: The less-cooperative *G. aggregatum* retained significantly more P in form of PolyP, than the cooperative AM fungus *G. intraradices* 61
Figure S7: Long-chain PolyP pools of cooperative and less-cooperative AM fungi 62
Figure S8: Model showing carbon and phosphate exchange in roots colonized with a cooperative or less-cooperative AM fungus 62
Figure S9: Response of the qPCR signal to DNA template dilutions 63
Figure S10: Calibration curves for the qPCR assays 64
Figure S11: MySQL table structure of PHYMYCO-DB 112
Figure A1: Experimental set-up 89
Figure E1**:** Effect of *F.candida* presence and absence on the mean dry weights of fungal hyphae collected outside the meshbags 94
Figure S12: Plot numbering of the e120 experiment in Cedar Creek LTER 137
Figure S13: Strategy to analyse the diversity of the root associated microorganisms 138

List of tables:

Table 1: Plant species present in the e120 experiment 118
Table 2: List of the plots sampled 119
Table 3: OTUs list 136
Table S1: qPCR marker 65
Table S2: Results of cross-specificity assay under optimized cycling conditions for each AM species-specific qPCR marker 66
Table S3: Detection limits and minimal detectable target gene concentrations of the three qPCR assays 67
Table B1: qPCR markers for specific quantification of *Glomus intraradices* and *G. custos* 90
Appendix C: Summary table of results and statistics 92
Appendix D: Comparison of ANOVA and GLM analyses for biomass measurements and secondary metabolites in roots and shoots. 93

List of Diagram:

Diagram 1: Diagram representing interspecific relationships 14

Instandhouding van samenwerking en diversiteit in de mycorrhiza-symbiose

Samenvatting

Mutualistische relaties tussen planten en arbusculaire mycorrhiza-schimmels (AMF) zijn uitermate wijd verbreid (ongeveer 80% van de landplanten worden door deze organismen gekoloniseerd) en oud (meer dan 450 miljoen jaar). Deze symbiose is een essentiële factor in het functioneren en de productiviteit van ecosystemen; ze is ook sterk betrokken bij de kringloop van twee sleutelelementen, fosfor en koolstof. In de huidige context van verlies van biodiversiteit is de instandhouding van dit mutualisme erg belangrijk geworden.

Het doel van dit proefschrift was om de stabiliteit van het plant-schimmel-mutualisme beter te begrijpen. Ik richtte mijn aandacht allereerst op de uitwisseling van nutriënten, waarbij ik testte of de gastheerplant en de schimmelsymbiont in staat zijn om onderscheid te maken tussen verschillende potentiële partners en meer middelen toe te wijzen aan partners die meer nutriënten aanleveren. Vervolgens bestudeerde ik de mogelijkheid dat de gastheerplant betrokken is bij de levering van secundaire metabolieten aan de schimmelhyfen. We ontwikkelden een nieuwe hypothese, namelijk dat de door de plant geleverde chemische bescherming positief gecorreleerd is met het niveau van samenwerking (d.w.z. nutriëntenlevering) door de wortelsymbionten.

Vervolgens bewoog ik me van het individuele niveau naar het niveau van de levensgemeenschap door het effect te bestuderen van een afname van de plantendiversiteit op de diversiteit van wortelsymbionten. Hiervoor gebruikte ik moleculaire analyses en innovatieve methodes, zoals het grootschalig uitlezen van DNA. Om de studie van de verkregen DNA-sequenties van mycorrhiza-schimmels en andere schimmels te vergemakkelijken, werkte ik samen met collega's aan de oprichting van een gegevensbank getiteld "Phymyco-DB", die publiekelijk opengesteld werd in 2012. Ten slotte bediscussieer ik de implicaties van het mycorrhiza-mutualisme in de context van landbouwsystemen en ik stel nieuwe wegen voor om zulke systemen te beheren.

Dit promotieonderzoek biedt nieuwe inzichten in de vraag hoe de interacties tussen planten en AM-schimmels werken en hoe ze ecologische en evolutionaire processen vormgeven in natuurlijke en landbouwecosystemen. Deze inzichten zijn van doorslaggevend belang bij de ontwikkeling van een meer ecologisch verantwoorde intensieve landbouw. Dit project heeft nieuwe kennis ontwikkeld en nieuwe visies in beeld

gebracht ten aanzien van het verlies van soortendiversiteit bij planten en de gevolgen daarvan voor de stabiliteit van AM-symbioses. Omdat arbusculaire mycorrhiza-schimmels essentieel zijn bij ecosysteemprocessen en het behoud van bodemvruchtbaarheid zou dit werk een belangrijke invloed moeten hebben in (i) het bodembeschermingsbeleid, (ii) het onderzoek naar plantenveredeling, en (iii) het ontwerp van duurzame landbouwsystemen.

Sleutelwoorden: mutualisme, planten, arbusculaire mycorrhiza-schimmel, diversiteit, levensgemeenschap, evolutie, samenwerking, ecosysteemfunctioneren, koolstofallocatie, secundaire metabolieten, SSU rRNA gen, grootschalig uitlezen van DNA.

Summary

The mutualism between plants and arbuscular mycorrhizal fungi is extremely widespread (~ 80% of plants are colonized by these organisms) and ancient (over 450 million years ago). This symbiotic relationship is an essential component of healthy ecosystem functioning and productivity, and is strongly involved in the cycle of two key elements: phosphorus and carbon. Maintaining this mutualism has become especially important in the current context of a biodiversity loss. One goal of this thesis was to understand the stability of the mutualism. I first focused on nutrient exchange, testing whether plant host and fungal symbionts are able to discriminate among partners, and allocate more resources to those individuals providing more nutrients. I then explored the possibility of the host-plant involvement in the protection of mycorrhizal symbionts via a transfer of secondary metabolites into fungal hyphae. We introduced a new hypothesis suggesting that chemcial protection from the plant is positively correlated with the level of cooperation (i.e. nutrient transfer) of the fungal symbiont. I then moved from the individual to the community by studying the effects of decreasing plant diversity on the diversity of root symbionts. To this aim, I utilized molecular analyzes and innovative tools, such as high throughput sequencing. To further facilitate the study of the obtained sequences and other fungal sequences, I worked with colleagues to create a database 'Phymyco-DB' which was released to the public in 2012. Finally, I discuss the implication of the mycorrhizal mutualism in the context of current agricultural systems and propose new trajectories to manage these systems.

This PhD project provides new insights on how plant and AM fungi interactions work and how they shape ecological processes and evolutionary trajectories in natural and agricultural ecosystems. These points are of major importance to develop a more ecologically intensive agriculture. The project has provided new knowledge and perspectives on the loss of plant diversity, and its consequences for AM symbiosis stability. As arbuscular mycorrhizal fungi are essential in ecosystem processes and soil fertility maintenance, this work should have a broad impact in (i) the soil protection policy, (ii) the research on plant breeding and (iii) the design of sustainable agricultural systems.

Key words: mutualism, plant, arbuscular mycorrhizal fungi, diversity, communities, evolution, cooperation, ecosystem functioning, carbon allocation, secondary metabolites, SSU rRNA gene, amplicon mass sequencing.

Résumé

Le mutualisme entre les plantes et les champignons mycorhiziens arbusculaires est extrêmement répandu (~ 80% des plantes sont colonisées par ces organismes) et ancien (il y a plus de 450 millions d'années). Cette relation symbiotique est une composante essentielle du fonctionnement des écosystèmes et de leur productivité, et est fortement impliquée dans le cycle de deux éléments clés: le phosphore et le carbone. Le maintien de ce mutualisme est devenu particulièrement important dans le contexte actuel de perte de biodiversité. Un des objectifs de cette thèse était de comprendre la stabilité de ce mutualisme. L'accent a tout d'abord été mis sur les échanges de nutriments impliqués dans cette symbiose, en testant si la plante hôte et les symbiotes fongiques sont capables de discriminer leurs différents partenaires, et d'allouer davantage de ressources aux partenaires fournissant plus de nutriments. La possibilité de l'implication de la plante hôte dans la protection des symbiotes mycorhiziens via un transfert de métabolites secondaires dans les hyphes a ensuite été étudiée. Nous avons alors pu émettre une nouvelle hypothèse suggérant qu'une protection impliquant des métabolites secondaires venant de la plante serait positivement corrélée avec le niveau de coopération (à savoir le transfert des nutriments) du champignon symbiotique. L'échelle d'étude est ensuite passée de l'individu à la communauté en étudiant les effets de la diminution de la diversité végétale sur la diversité des symbiotes racinaires. Pour ce faire, des analyses moléculaires et des outils novateurs ont été utilisés, tels que le séquençage à haut débit. Pour faciliter l'étude des séquences obtenues, une base de données 'Phymyco-DB' rendue publique en 2012 a été utilisée. Enfin, je discute de l'implication du mutualisme mycorhizien dans le contexte des systèmes agricoles actuels et propose de nouvelles trajectoires pour gérer ces systèmes.

Ce projet de thèse apporte un nouvel éclairage sur la façon dont fonctionnent ces interactions entre les plantes et les champignons MA et sur la manière dont ils façonnent les processus écologiques et les trajectoires évolutives dans les écosystèmes naturels et agricoles. Ces points sont d'une importance majeure pour le développement d'une agriculture plus écologiquement intensive et durable. Le projet a fourni de nouvelles connaissances et perspectives sur la perte de la diversité végétale et ses conséquences pour la stabilité de la symbiose MA. Comme les champignons mycorhiziens arbusculaires sont essentiels dans les processus des écosystèmes et l'entretien de la fertilité des sols, ce travail devrait avoir un large impact dans (i) la politique de protection des sols, (ii) la recherche sur l'amélioration des plantes et (iii) la conception de systèmes agricoles durables.

Mots Clés: mutualisme, plante, champignons mycorhiziens à arbuscules, diversité, communautés, coopération, fonctionnement des écosystèmes, allocation de carbone, métabolites secondaires. SSU rRNA, séquençage de masse.

General introduction

I. Relationships among living organisms

Ecosystems are continuously changing under the action of ecological and evolutionary forces. In addition to abiotic factors, biotic determinant, can dramatically affect the capacity of a given organism to survive in an ecosystem. A pathogen or a competitor may negatively impact individuals thereby causing changes in ecosystem composition, and these intra- and interspecific relationships between species mediate ecosystem dynamics (Brown *et al.,* 2001). A high degree of ecosystem complexity is generally explained by a great complementarity in resource use via niche differentiation or facilitation (Loreau & Hector, 2001).

Like biotic factors, species can impact their own habitat via modification of abiotic factors. For example, particular plants can induce soil acidification and modify availability of nutrients through the palatability and biodegradability of the organic matter they produce (Wilcke *et al.,* 2000).

Relationships among organisms are numerous. They can include cooperative, neutral, antagonistic and agonistic behaviours. Antagonisms are generally characterized by parasitism, competition, and predation, while cooperation is defined by positive, mutualistic interactions (Rico-Gray, 2001) (Diagram 1). These interactions can be specialized or opportunistic. Symbiosis generally involves intimate associations between two or more organisms and is a major driver of ecosystems functioning (e.g. Margulis, 1992). In this thesis I consider the eco-evolutionary dynamics of symbiosis.

1. Symbiosis: definition

Symbiosis, according to the Oxford dictionary, is an “interaction between two different organisms living in close physical association, typically to the advantage of both”. Close associations between different species of organisms have been known since the end of the 19th century. Cienkowski (1871) observed and demonstrated that *Radiolaria* (a group of marine protists) harboured small dinoflagellate algae in their extracellular coat, which were also able to live outside the organism. At about the same time, Frank (1877)

highlighted relationships between plant roots and fungi in an association called mycorrhiza. Frank defined this association between the two partners as “zusammenleben”, or “living together” and coined the term “symbiotismus” to differentiate it from parasitism. In 1879 the word “symbiosis” was proposed by de Bary, who was working on lichens, to explain a close interaction between at least two different organisms. This definition now covers a wide range of interactions ranging from mutualism to parasitism. Van Beneden (1875) had already described mutualism, commensalism and parasitism but had found it difficult to define the limits between the categories. In this thesis, I use de Bary’s definition of symbiosis (i.e. a close interaction between at least two different organisms and that benefits at least one of the species) because symbiosis generally involves more than two partners with behaviours ranging from mutualism to parasitism (Diagram 1).

		Species 1		
		Positive	Negative	Null
Species 2	Positive	Mutualism		
	Negative	Parasitism Predation	Competition	
	Null	Commensalism	Amensalism	Neutral

Diagram 1:

Diagram representing the six interspecific relationships ranging from mutualism (positive vs positive) to competition (negative vs negative) depending on the positive, negative or neutral effect of each species on the other. This diagram is modified from Principles of Animal Behavior. (http://eebweb.arizona.edu/animal_behavior/lycaenids/lycaen2.htm)

Margulis (1992) argued that symbiotic relationships between species (especially mutualism) have had (and continue to have) a major impact on the evolution of organisms and thus on ecosystem functioning. Mutualistic symbiosis can increase the functioning of organisms compared to their stand-alone state, allowing symbiotic organisms to colonize new ecosystems to which they were not previously adapted.

2. Theory: Evolutionary forces and the symbiosis dilemma

Mutualistic associations are diverse and are found in all kingdoms of the tree of life. All Eukarya are involved in a specific form of mutualistic relationship, the intracellular inclusion of an alphaproteobacterial cell from which the mitochondrion evolved. This mutualistic interaction is assumed to have led to the emergence of Eukarya (e.g. Cavalier-Smith, 2002). Other mutualisms, such as between plants and mycorrhizal fungi, are thought to have lead to land colonization by plants (e.g. Selosse & Le Tacon, 1998). From an evolutionary point of view, the evolution of mutualism, and especially its establishment, is difficult to explain (Cameron *et al.,* 2008; Davitt *et al.,* 2010; Frederickson *et al.,* 2012). Natural selection will consistently favour organisms that are selfish; both partners in a trophic interaction are assumed to be subject to selection that will to maximize their gains from the relationship, while giving as little as possible in return. So why does cooperation not break down?

If the fitness of the host is tightly aligned with the fitness of the symbiotic partners, this could help select for cooperation (i.e. vertical transmission, see below). However, multiple partners are often involved simultaneously in symbiosis within a single host (e.g. mycorrhiza and nitrogen-fixing nodules in plants, the gut microbiome in animals). In these cases, selection for defection from cooperation (i.e. ‘cheating’) will be even stronger, and can increase the instability of the symbiosis (e.g. Denison *et al.,* 2003; Kiers & Denison, 2008). This is because cooperative partners that supply their hosts with resources indirectly aid competing strains (including non-cooperative ones) colonizing the same individual. This means individuals can experience a selective benefit from providing few resources. In social sciences, this is known as the “tragedy of the commons” (Hardin, 1968). To prevent defection in mutualisms, hosts have evolved mechanisms to better control their symbionts. These are discussed below.

3. Key mechanisms contributing to stability of 'mutualistic' symbiosis

Several mechanisms have been proposed that may stabilize the initiation of mutualistic relationships. These include partner choice, vertical transmission, enslavement and lineage suicide (Sachs *et al*., 2004; Bright & Bulgheresi, 2010).

a. Partner choice

Partner choice is a mechanism that allows hosts to identify and extract high quality partners from the environment (Golubski & Klausmeier, 2010; Archetti *et al.,* 2011). To be efficient, the host partner needs to be exposed to a range of potential symbionts. Then based on signals that indicate quality and level of cooperation, hosts can choose the most appropriate partner. This type of mechanism has been shown to operate in some cases in the *Rhizobium*-legume symbiosis (e.g. Simms & Taylor, 2002), allowing the legume host to select particular genotypes of rhizobial symbionts. Similar choice mechanisms have been reported to be utilized by coral, fish and shrimp hosts with regard to their respective symbionts (Lewis & Coffroth, 2004; Jaafar & Hou, 2012). Also in ants and aphid mutualisms a mechanism based on partner choice has been demonstrated (Verheggen *et al.,* 2012). In many animals, the composition of the gut microbiome is controlled, at least in part, by host immunity (Boehm, 2012); this can be viewed as a form of partner choice. However in any partner choice, there is still a potential for the symbiont to cheat, for example by evolving deceptive signalling mechanisms (Simms & Taylor, 2002), especially if it has a much shorter generation time than the host.

b. Vertical transmission

Vertical transmission is another means of ensuring that partners remain cooperative. This strategy relies on the vertical transmission of symbiont(s) across host generations. This aligns the reproduction of the host with the reproductive success of its symbiont. It has been found in the transmission of fungal endophytes in some grasses and the transmission of gut symbionts in termites (Noda *et al.,* 2007; Omacini *et al.* 2012). In aphids, a limited number of *Buchnera* are transmitted from mother to the progeny through the transfer of bacteriocytes to the embryo (Koga *et al.,* 2012). These *Buchnera* are mutualistic symbionts which are involved in parthenogenetic reproduction of the insect. It has been hypothesized that *Buchnera* strains are filtered via the reproduction process, resulting in a kind of purging of low-quality lineages (Bright & Bulgheresi, 2010).

c. Obligate dependency

A third mechanism to prevent symbionts from defecting from cooperation is via an

increase in the dependency of the symbiont on the host. By increasing the dependence of the symbiont on the host, the host gains greater control over the interaction. Dependency can be so intense that the genome size of symbiont is reduced (McCutcheon & Moran, 2012), leading to loss of independent functioning. The most classic example is the case of mitochondria and chloroplasts which evolved, respectively, from purple non-sulphur bacteria and cyanobacteria, enslaved by their host cells (e.g. Margulis, 1993).

While these mechanisms have been shown to stabilize cooperation in other mutualistic systems, the plant-mycorrhizal mutualism cannot be explained by these host mechanisms. Here, I introduce the plant-mycorrhizal mutualism, and ask how this relationship is stabilized.

II. Symbiosis between plants and fungi

Plant mutualisms are incredibly important in ecosystem productivity and functioning. The best known examples are (i) the nitrogen fixing root nodules of *Rhizobium* and *Frankia,* (ii) mycorrhizal fungi, and (iii) endophytic fungi. The most common of these mutualisms at the planetary scale is the mycorrhizal relationship. There are three main types of mycorrhizal mutualisms: arbuscular mycorrhiza (AM), ecto-mycorrhiza and ericoid-mycorrhiza (Trappe, 1987; Brundrett, 2009). Arbuscular mycorrhizal fungi (i.e. Glomeromycota) arguably constitute an extremely widespread mutualism form on earth (Smith & Read, 2008), and include the majority of plant species, including bryophytes, pteridophytes, gymnosperms and angiosperms (Johnson & Gehring, 2007).

1. The AM symbiosis

a. History, taxonomy

The AM symbiosis emerged and evolved more than 450 million years ago (Redecker *et al.,* 2000; Humphreys *et al.,* 2010). Approximately 80% of land plants are colonized by AM fungi (Smith & Read, 2008). AM fungi have a global distribution in terrestrial ecosystems, and are also found in oligotrophic lakes in association with isoetid species (Møller *et al.,* 2013). The AM fungal symbiosis is believed to date back to the very origin of the evolution of land plants and is believed to be a major factor contributing to plant success in terrestrial ecosystems. The AM symbiosis is a striking example of a

mutualism that does not follow the above strategies for stabilizing cooperation (i.e. paragraph I.2.).

AM mutualism emerged early and was likely responsible for land colonization during the Devonian. This mutualism is widespread and highly successful. To explain its stability, we hypothesize that plants can detect and control low-quality AM fungal symbionts via differential allocation of carbon resources.

We also hypothesize that plants protect the symbiont against fungivores by transferring protective secondary metabolites to the fungus.

The first species of *Glomus, G. microcarpus* and *G. macrocarpus,* were described by Tulasne & Tulasne in 1844. The *Glomus* genus was associated with *Endogone* in the *Endogonaceae (Zygomycota)* family because of the similarity of their respective spores. In 1851, Tulasne & Tulasne moved *G. microcarpus* and *G. macrocarpus* into the *Endogone* genus. In 1974, Gedermann & Trappe, considered *Glomus* as a genus on its own and placed AM fungi in the genera *Glomus*, *Acaulospora*, *Gigaspora* and *Sclerocystis*, still in the *Endogonaceae* family *(Zygomycota)*. All AM fungi were then brought together in a new order within the Zygomycota, Glomerales, by Morton & Benny (1990).

Until the 1990s, all taxonomical classifications of AM fungi were based on phenotypic features and morphological descriptions of spores. Spore “walls” were/are particularly well-studied because of their diversity. At that time, the first molecular analyses based on ribosomal SSU gene sequences were being used to determine phylogenetic relationships between AM fungal taxa. All these taxa were classified in the polyphyletic phylum of the *Zygomycota* until 2001. On the basis of SSU rRNA gene analyses showing monophyly of Glomerales, Schüßler *et al.* (2001) suggested the removal of AM fungi from *Zygomycota* and raising the Glomerales to phylum level and renaming it *Glomeromycota* (Figure 1).

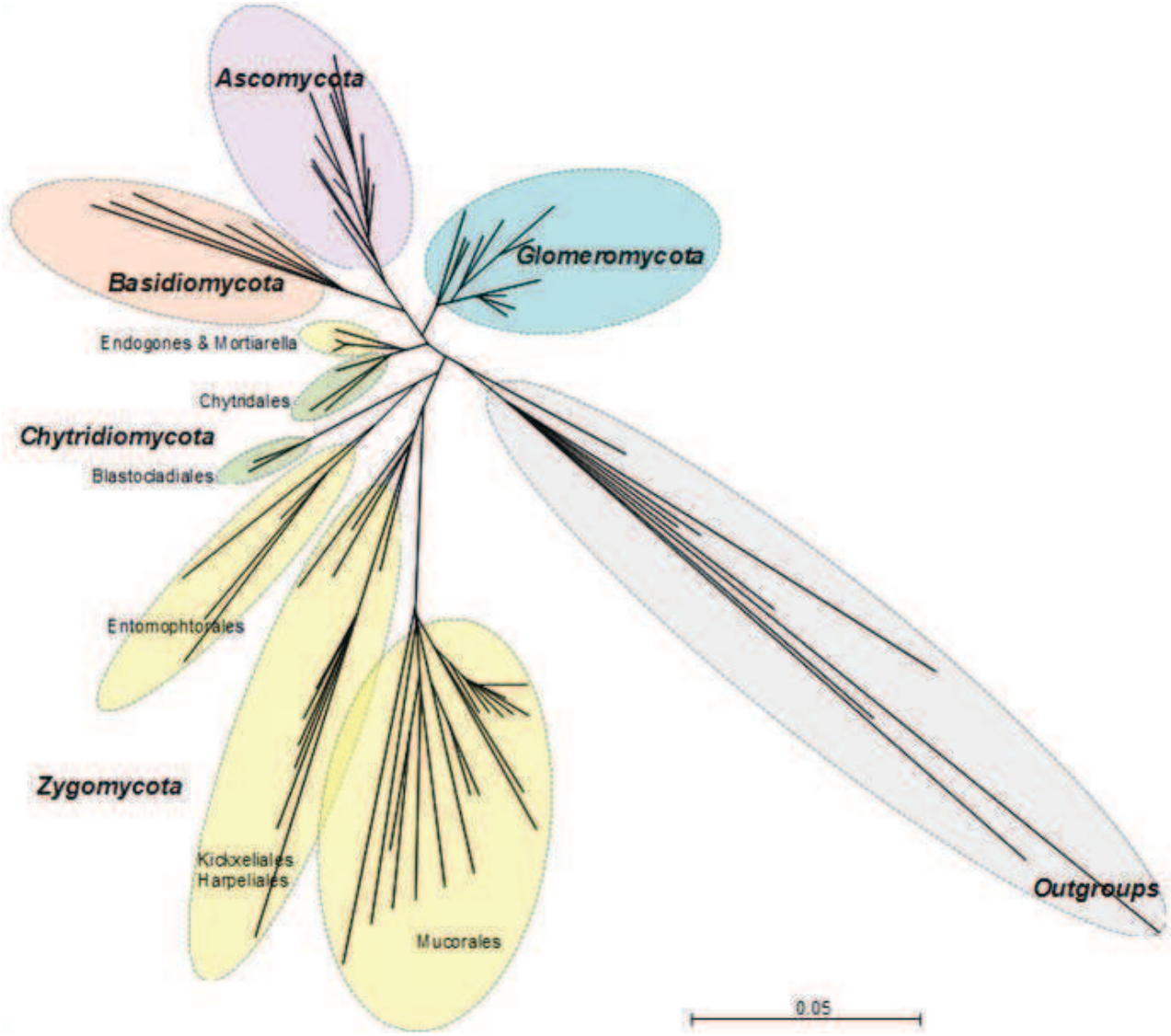


Figure 1:
Fungal phylogenetic tree showing the relative position of the Glomeromycota phylum within the evolutionary 'landscape' of other fungal phyla. This phylogenic tree is based on SSU rRNA gene sequences. Unlike Ascomycota, Basidiomycota and Glomeromycota,, Chytridiomycota (green) and Zygomycota (yellow) are polyphyletic. This figure is modified from Schüßler *et al.* (2001). The scale bar represents the number of substitutions per site.

The *Glomeromycota* phylum now consists of one class: *Glomeromycetes*, and five orders: Glomerales, Diversisporales, Gigasporales, Archaeosporales and Paraglomerales (Oehl *et al.,* 2011). These orders contain in total 14 families and 26 genera (Figure 2). Most likely, only a very small proportion of AM fungal species have been described so far (e.g. Opik *et al.,* 2006).

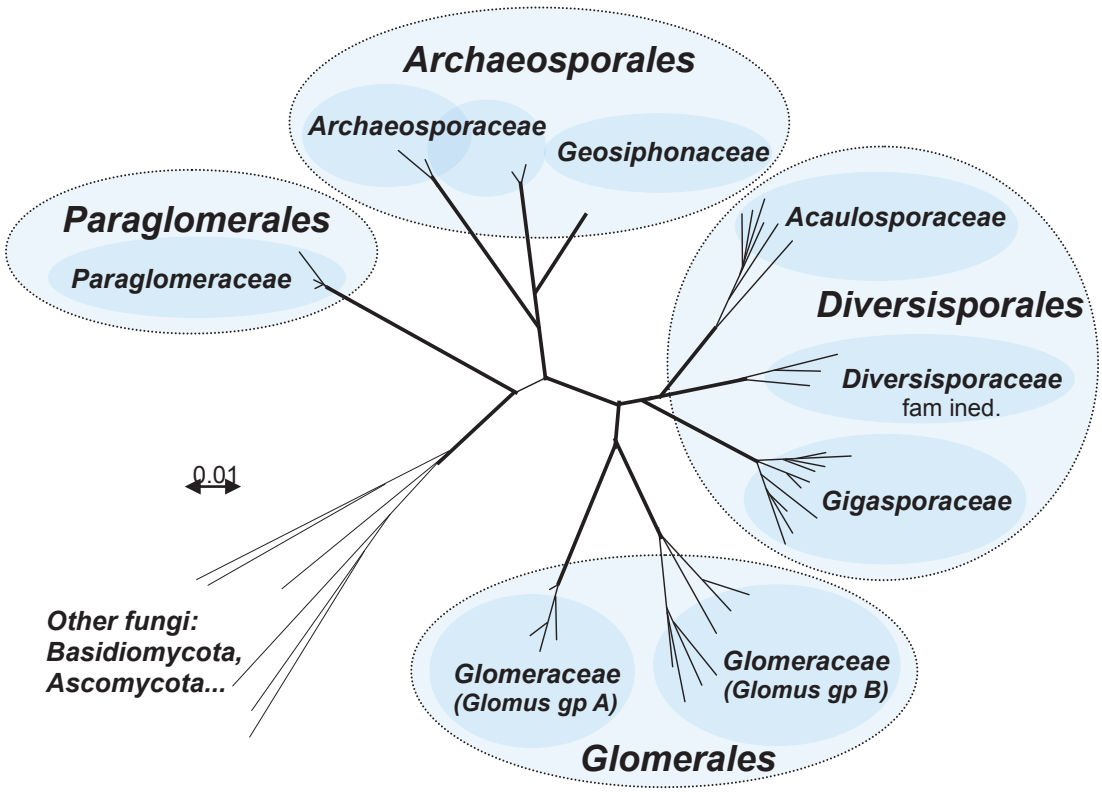


Figure 2:

Phylogenetic tree of the Glomeromycota phylum based on SSU rRNA gene sequences presenting four orders and eight families. This figure is modified from Schüßler *et al.* (2001); the Gigasporacaea order described in Oehl *et al.* (2011) is not shown in this figure. The scale bar represents the number of substitutions per site.

b. Toward a molecular diagnosis of fungal diversity?

Molecular detection of the AM fungi colonizing roots has led to the discovery of many previously unknown species. However, the diversity of Glomeromycota is still poorly understood (e.g. Vandenkoornhuyse *et al.,* 2002a). A major issue that remains is to determine the formal fungal taxonomic codes (i.e. markers) to designate taxa known only from molecular signatures (Hibbett *et al.,* 2011). Further problems include (i) dual nomenclature existing for pleomorphic fungi (ii) the species name can include a complex of different organisms having similar morphologies (Vandenkoornhuyse & Leyval, 1998) (iii) a known fungus such as *Glomus mossae* was renamed *Funneliformis mossae* (Schüßler & Walker, 2010) without this modification being published in a peer-reviewed journal. These

aspects lead directly to problems in sequence annotations (i.e. synonymous names for a given sequence or reciprocally one name for a variety of sequences) and emphasize the need for significant changes. There is also an urgent need to limit contamination and the propagation of mistakes in sequence annotations in public databases. Sequences must be well identified and annotated to ensure the correct diagnosis of community diversity and production of robust phylogenies. In this context, the need to filter public sequence databases by stringent curation has become a fundamental issue. I addressed this problem by contributing to the development and curation of the Phymyco database (see chapter IV).

Molecular analyses based on SSU rRNA gene sequences have been successfully used to detect the whole fungal community colonizing the roots of a plant (Vandenkoornhuyse *et al.*, 2002a), and also to detect AM fungi specifically (e.g. Helgason *et al.*, 1998). This molecular target, shared by all living organisms, has both highly conserved and lineage-specific variable sequences. It contains a high quality phylogenetic signal that can be used to determine phylogenetic species and requires implicit adoption of the phylogenetic species concept (Mishler & Brandon, 1987). However many mycologists are still using the Internal Transcribed Spacer (ITS), which is a highly variable region in the cluster of rRNA genes. This molecular marker is used as a barcode that allows the identification of a given species if a query sequence has a very close relative and properly annotated sequence. However, ITS contains poor phylogenetic information which makes the correct construction of phylogenies difficult, even impossible. This molecular target is even more difficult to use in AM fungi, as compared to other fungal groups, because the genomes of AM fungi can exhibit considerable variation among their ITS copies (e.g. Sanders *et al.*, 1995). Thus, the AM fungal diversity should be studied using appropriate markers meeting the criteria for a reliable identification of sequences.

c*. Description, characteristics of arbuscular mycorrhiza*

AM fungi are biotrophs, multinucleate and ‘asexual’ although evidence for recombination exists (e.g. Vandenkoornhuyse *et al.*, 2001). Their life cycle is poorly understood. Different specific structures of AM fungi are formed inside and outside roots (Figure 3).

The mutualism begins with spore germination (Figure 5). Spores produce hyphae that grow through the soil toward the host plant (Giovanetti *et al.*, 1993). When hyphae come into contact with exudates released from the roots, branching factors lead to intense

hyphal ramification (Figure 5). These branching factors have been shown to be sesquiterpene lactones and more especially strigolactones (compounds also released in the presence of parasites) (Akiyama *et al.*, 2005; Ruyter-Spira *et al.,* 2013). The hyphae then colonize the root surface.

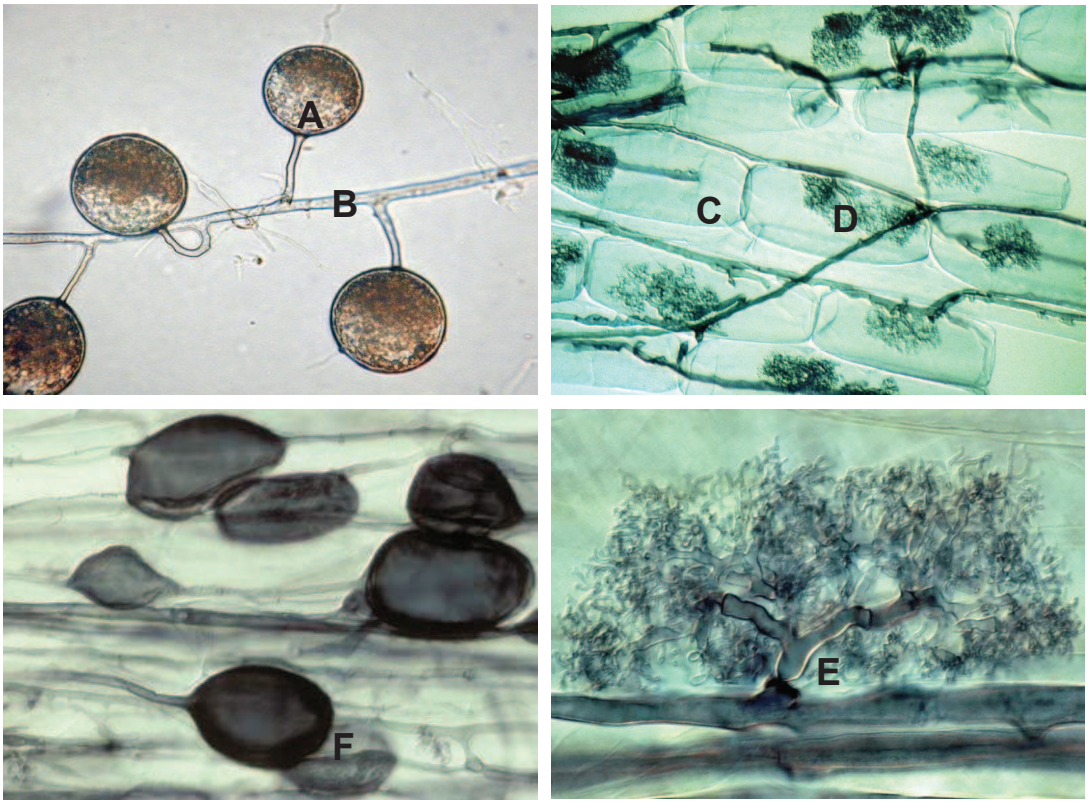


Figure 3:
Different structures of arbuscular mycorrhizal fungi: (A) spores (10 to 600 µm), (B) non septate hyphae (~5 to 10 µm), (C) arbuscules that are found in (D) root cells. (E) detail of an arbuscule. In this figure (F) vesicles constituting lipid storage structures are also shown. (Photo credits from left to right and top to bottom: McGee 1986; Symbiom http://bohdana77.wix.com/vttrial1#!mycorrhiza; Bundrett, 1984; Bundrett, 2008; visualized by optical microscopy).

As the fungus enters into contact with the root surface, cell to cell recognition takes place between the two organisms leading to the formation of a swollen hyphal structure or appressorium (figure 5). At this point, the root cell nucleus migrates to the contact area, opposite the appressorium. This latter penetrates the cell following the nucleus path (Genre *et al.,* 2005). The cell modifications allowing AM fungal infection are triggered by a

fungal compound, the *Myc*-factor (Albrecht *et al.*, 1999). Passage through the outer layers of the root involves lytic enzymes. Hyphae circulate in the intercellular spaces or directly through cells (Parniske, 2004).

In the inner cortex, the appressorium penetrates the cell wall and starts to form highly branched haustoria (Figure 5). These tree like structures, called arbuscules, cause multiple invaginations of the plant cell membrane. The arbuscules are completely surrounded by the periarbuscular plasma membrane, which isolates them from the root cell protoplast (Paszkowski, 2006). The root cell structure is thus deeply modified by arbuscule formation. In addition to nucleus migration toward the centre of the cell, due to reorganization of the cytoskeleton (Genre *et al.,* 2005), there is also fragmentation of the central vacuole and increased transcriptional activity. Nutrient exchanges between plant and fungi take place at the level of the arbuscules. These structures collapse after a few days of activity.

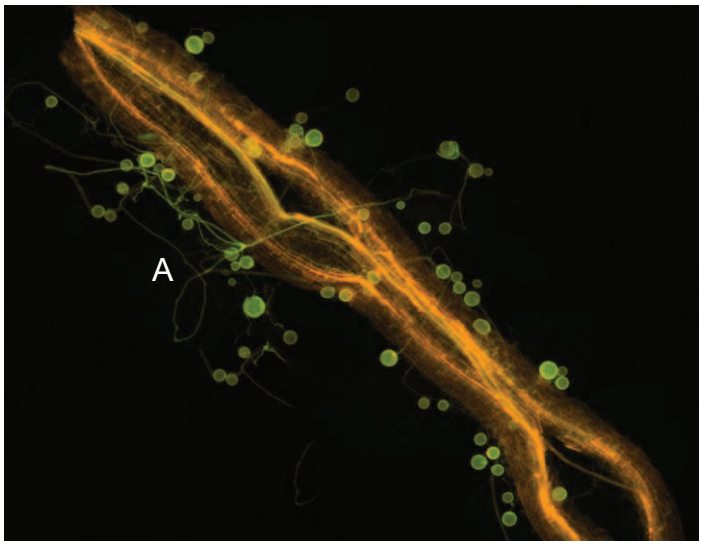


Figure 4:
Autofluorescence micrograph of arbuscular mycorrhizal fungi (in green) colonizing roots (in brown) of its host-plant, *Medicago truncatula*. (A) are the hyphae constituting the extraradical mycelium (ERM). (Photo credit : Jan Jansa)

Simultaneously to arbuscule formation, runner hyphae (the extraradical mycelium, ERM; Figure 4) are produced in the soil. These explore the soil and acquire resources. The ERM are then able to form new infection units. One plant can be colonized by several AM fungal species within the same root and one AM fungus can colonize several plant species to form a complex network (Figure 4). AM fungi display different colonization

strategies depending on the AM fungal family (Hart & Reader, 2002). Whereas voluminous external hyphae are produced by the Gigasporaceae for soil colonization (6 to 9 m/cm3), much fewer are produced by the Acaulosporaceae and Glomeraceae (1 to 2 m/cm3).

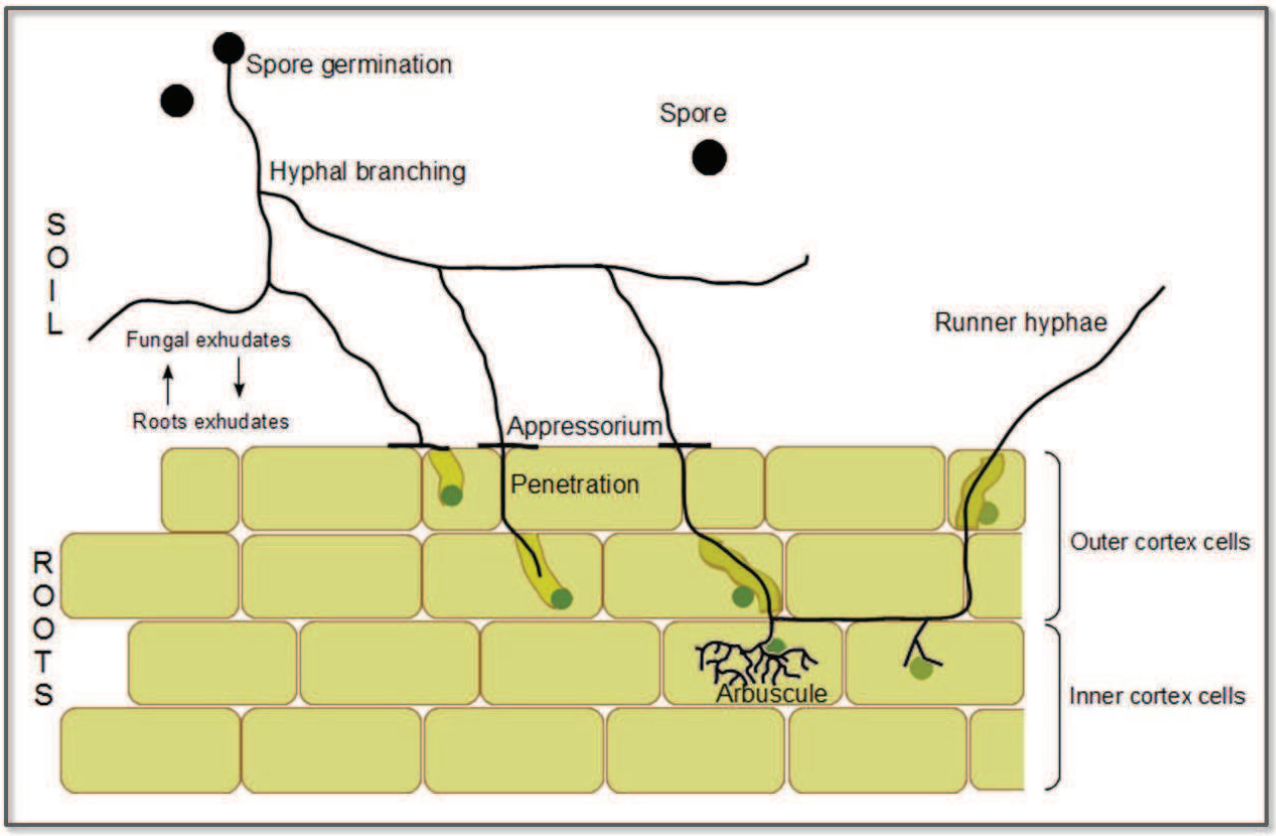


Figure 5:
The different steps of root colonization by an AM fungus. Modified from Bonfante & Genre, 2010.

d. Nutrient exchanges

AM fungi are known to improve plant mineral nutrition via nutrients collected in their hyphal network. Each hypha is of very small diameter, allowing for extremely efficient soil exploration. The hyphal network improves plant access to water, and can provide nutrients such as nitrogen, zinc and copper. However the major nutrient provided to the plant is phosphorus. Up to ~70 % of all plant phosphorus can be delivered by AM fungi (Smith *et al.,* 2003). AM fungi collect mineral nutrients and water from soil via transporters. Phosphorus enters the hyphae via phosphate transporters, then circulates through the hyphae towards the arbuscules where they are deposited as polyphosphate chains. These chains cannot be assimilated by the root cells but are broken down into monophosphates

by polyphosphatase in the arbuscules. These monophosphates are then transferred through the periarbuscular membrane toward the root cells by P transporters (Parniske, 2008).

In exchange for these services, AM fungi receive about 20% of photosynthates from the plant (Bago *et al.,* 2000; Figure S8). Photosynthesized sugars and polyolisides enter the periarbuscular space, where they are then split into glucose and fructose. These hexoses are taken up via hexose transporters into the fungus where they are converted to the disaccharide trehalose and to lipids (Bonfante & Genre, 2010; Parniske, 2008)

2. No host specificity but host-plant preference

Although roughly 270,000 known plant species are colonized by AM fungi (Smith & Read, 2008), fewer than 200 species of AM fungi have been described so far. This unbalance in species number may simply indicate that each AM fungal species has a wide range of plant hosts. This low specificity between the host-plant and AM fungi has been known for a long time (e.g. review of Gianinnazzi-Pearson, 1984) and confirmed repeatedly (e.g. van der Heijden *et al.*, 1998; Santos *et al.*, 2006). However, AM fungi are not randomly distributed among host-plants species (Eom *et al.,* 1999). Repeatable patterns of AM fungal community have been found colonizing a given host-plant species at a given location (Vandenkoornhuyse *et al.,* 2002b, 2003) suggesting a preferential association likely as a result of host-plant fitness (Vandenkoornhuyse *et al.*, 2002a). It is also well-known that the growth of the host-plant varies depending on the symbiont, (e.g. van der Heijden *et al.*, 1998), and that different host plants are colonized by different AM fungal symbiont communities (Vandenkoornhuyse *et al.,* 2002b, 2003). These observations have been interpreted to mean that there is some form of host-plant preference (Vandenkoornhuyse *et al.,* 2002b, 2003). Particular associations are likely determined by compatibility and success between one host and its AM symbionts, but also by stochastic effects such as spore dispersal (Verbruggen *et al.,* 2012). Hausmann & Hawkes (2009) have shown that when a given plant is grown alone in a soil containing several AM fungal species, the plant displays a host-plant 'specificity' but when this same plant is grown in the same soil with the same fungi but with other plants, there is no longer host specificity but instead a host preference.

Some plants (e.g. *Plantago lanceolata)* and some fungi (e.g. *Glomus intraradices*) are extreme generalists having a wide range of partners (Opik *et al.,* 2006; Helgason *et al.,*

2007; Maherali & Klironomos, 2007), while some associations are more specific such as *Glomus hoi* which is almost always found in *Acer pseudoplatanus* roots (Helgason *et al.,* 2002). Host-plant preference depends on the local availability of AM fungi propagules in soil (Scheublin *et al.,* 2004). Different signals are triggered for the mutual recognition of both partners through root exudates, such as branching factor (strigolactones), that favour colonization and proliferation (Giovanetti *et al.,* 1994) of certain types of AM fungi, and *via* fungal components such as glomalin and M*yc* factor (Reinhardt, 2007; Requena *et al.,* 2007; Hartmann *et al.,* 2009). These specific compounds stimulate colonization and hyphal propagation. As shown by Teutsh-Hausmann *et al.* (2010), AM fungal communities in plant roots can also be controlled by the order of plant establishment, the actual plant host and the vicinity of this plant. Plant/AM fungi associations can also vary with environmental conditions and plant colonization by an AM fungus will also depend on the season (Santos Gonzales *et al.,* 2007). Indeed root and soil colonization by different AM symbionts exhibits both temporal and spatial variation. Not all AM fungi species are active at the same time (Smith *et al.,* 2000). Soil disturbance (Schnoor *et al.,* 2011) and soil physicochemical properties may influence the host-plant preference (Martínez-García & Pugnaire, 2011), as the efficiency of AM fungi and thus their ability to colonize roots will be impacted by the phosphate concentration (Ehinger *et al.,* 2009).

AM fungal species/genotypes can differ dramatically in terms of nutrient acquisition, plant pathogen protection, drought resistance, etc (Bhattacharjee & Sharma, 2012; Wilson *et al.,* 2012). Hosts may benefit from this diversity and preferentially associate with different fungal strains depending on biotic context.

3. Importance of AM symbiosis in ecosystems

a. Importance for plant development and reproduction

AM symbioses can be extremely important in ecosystem functioning and processes via different pathways (Rillig, 2004). On the global level, the AM fungal symbiosis is responsible for massive nutrient transfer. It is a mutualism 'that helps feed the world' (Marx, 2004; Duhamel & Vandenkoornhuyse, 2013, *in revision*) by playing a fundamental role in crop growth. In addition to plant mineral nutrition, AM fungi are involved in plant phytoprotection (Azcon-Aguilar & Barea, 1996; Smith *et al.,* 2010). AM fungi colonization can elicit plant defence mechanisms (Abdel-Fattah *et al.,* 2011; Jung *et al.,* 2012) and have beneficial effects on plant stress, e.g., resulting from the presence of heavy metals in

the soil (Pallara *et al.,* 2013).

In addition to having a profound impact on plant growth, AM fungal colonizers also affect plant sexual and asexual reproduction (Varga, 2010). As reviewed by Koide & Dickie (2002), their presence increases plant reproduction by acting on both male and female functions especially when there is phosphorus deficiency. Streitwolf-Engel *et al.* (1997) have shown that AM symbionts are able to manipulate plant reproduction traits. AM fungi can modify the size, number of stolons and ramets in a clonal plant, and can facilitate seedling establishment in grasslands (van der Heijden *et al.,* 2004).

b. Impact of AM fungi on soil structure

The AM symbiosis has been shown to have a great impact on soil structure and stability. The hyphal network produced by AM fungi can bind the soil particles in stable aggregates. The amounts of such aggregates have been shown to be positively correlated with the length of the mycorrhizal hyphal network and roots (Jastrow *et al.,* 1998), In addition, AM fungi produce a glycoprotein, glomalin, which improves stability of soil aggregates. This compound is thought to be important for structuring and quality improvement of soil, by limiting erosion and water withdrawal (Rillig & Mummey, 2006).

c*. Fungal and plant community structure*

In addition to the effects of AM fungi on plant growth and fitness, AM fungi and plants can also affect each other's community structure, such as level of diversity. AM symbionts are able to influence the structure and productivity of plant communities (van der Heijden *et al.*, 1998; van der Heijden *et al.,* 2006a, 2006b). An increase in AM fungal species richness can have a positive effect on the health and productivity of the plant community (van der Heijden *et al.,* 2008; Wagg *et al.,* 2011). This could be linked to the multiple functions carried out by AM fungi. A larger range of functions resulting from the presence of different AM fungi species could lead to higher plant productivity (Maherali & Klironomos, 2007). The pool of AM fungi species in the soil, their development and the establishment of a hyphal network during the germination of a plant seed, have the potential to promote the establishment of other plants (Hausmann & Hawkes, 2009). AM fungi are thus playing an active role in plant species establishment and coexistence (van der Heijden *et al.*, 1998; Hart *et al.,* 2003). AM fungi are able to ‘relax’ plant-plant competition for their mutual benefit (Wagg *et al.,* 2011).

AM fungi can be involved in the colonization behaviour strategies of plants e.g.

those displaying allelopathy. AM fungi have been shown to expand the active area of emitted allelopathic compounds through the hyphal network (Barto *et al.*, 2011). Even more fascinating is the possibility of connections between plants through the hyphal network and the possible transfer of compounds from one plant to another across these 'hyphal bridges' (Barto *et al.,* 2009). The allelopathic compounds released by plants (Javaid, 2007) not only pass through the AM hyphae but may also regulate AM fungal growth.

Reciprocally, the plant community can impact the composition/diversity of the fungal community. Host plants play a role in growth and structuring of the AM fungal community (e.g. Bever *et al.,* 1996; Klironomos, 2002; Johnson *et al.*, 2003a). The host plant species can potentially affect the sporulation rate, growth and survival of its AM symbiont (Helgason *et al.,* 2009). Through the observed host-plant preference for a fraction of the available AM fungal community at a given location (Vandenkoornhuyse *et al.*, 2002b, 2003), host-plants can select for specific AM fungal community in soils.

It has been shown that the AM fungal communities of native plants can be modified or even suppressed by invasive species (Hawkes *et al.,* 2006; Callaway *et al.*, 2008). When several species of plant occur together, the effects of a given plant on its AM symbionts will be affected by the neighbourhood, suggesting that neighbourhood plants and their community composition are as important as the plant host itself in structuring the AM fungal community (Hausmann & Hawkes, 2009; 2010). The order of plant establishment is also likely to drive the AM fungal community trajectory (Hausmann & Hawkes, 2010). The first plants select a specific composition of developing AM fungal community, which in turn facilitates a particular trajectory for the establishment of new plants (Hausmann & Hawkes, 2010). This means that the host-plants in a particular location have the potential to impact the AM fungal community composition and diversity.

d. Link between AM symbiosis and ecosystem stability and productivity

AM fungi diversity has been shown to affect diversity and productivity of plant communities. Indeed, van der Heijden *et al.,* in 1998 and 2007 and Klironomos *et al.* (2000), demonstrated a positive correlation between fungal diversity and plant diversity, an increase of fungal taxa richness leading to an increase of plant species richness and productivity. Nevertheless, this positive correlation between plant and AM fungal species richness is likely more complicated. Johnson *et al.* (2010) showed that plant genotypic richness led to a modification of AM fungal community composition in roots, with a

decrease of AM fungal species richness as the number of plant genotypes increased. However, AM fungal species richness could be a poor proxy of functional diversity if we assume that a given AM fungus name corresponds to a polymorphism of ecological functions. At this point, the effects of host plant genotypes on the intraspecific diversity of AM fungi remain unknown. Plant intraspecific diversity (i.e. magnitude of genetic diversity within a species) and the related polymorphism in functional traits could also affect ecosystem functioning (Hughes *et al.,* 2008).

This AM fungal diversity affects plant diversity and productivity resulting in an increase of the organic matter diversity and quantity (Tilman 1982, 1997; Zak *et al*., 2003). As a consequence, this can have a positive impact on the diversity and functions of decomposers (i.e. higher functional complementarity), resulting in an improvement of nutrient cycling and thence a higher ecosystem productivity (i.e. Wardle *et al.*, 2003; Hughes *et al.,* 2008). As the AM fungal diversity increases, plants have access to a larger pool of functions. The coexistence of different plant species and their increasing diversity can be explained by their complementarity rather than competition.

The consequences of a decline in plant species richness & diversity for AM fungal species richness and diversity are not yet clearly known. We hypothesize that a decrease in plant diversity will have a negative impact on AM fungal species richness and diversity, and ultimately affect ecosystem productivity.

III. Objectives and approaches:

The objectives of this work were to study the evolution of cooperation in AM symbiosis and to analyse the link between plant diversity and fungal symbiont diversity.

Experiments were designed to investigate the evolution of cooperation. It is well-established that in the mycorrhizal mutualism, multiple symbiotic partners (of varying quality) can simultaneously colonize a single host. The mutualism is vulnerable to cheaters that benefit from colonizing a healthy host plant, but contribute little symbiotic benefit. We therefore ask how this mutualism is stabilized. We hypothesized that host plants are able to discriminate among the fungal communities in their root systems, and allocate the most carbon to the highest-quality symbionts. This hypothesis was tested utilizing an experimental Stable Isotope Probing (SIP) RNA approach under controlled inoculations (Chapter I).

The AM symbiosis exists in a web of multiple interactions. We were therefore interested in how adding additional organisms would affect cooperative dynamics. In a series of experiments, we included hyphal grazers (i.e. Collembola) to determine if fungal symbionts are chemically protected by their host plant. We hypothesized that a transfer of secondary metabolites from the plant to its AM symbionts would deter fungivore feeding. We used microcosms and controlled inoculations to determine whether AM symbionts received secondary metabolites from their host-plants as a protection against fungivores (Chapter II).

We developed and published for the public a sequence database to ensure proper analysis of these molecular data, avoid incorrect assignments, and provide high quality sequences for use as references, (see II.1.a). This database gathered reliable fungal SSU rRNA and EF1α sequences and permitted the determination, identification and phylogenetic analyses of fungi (Chapter III).

While we known that the AM symbiosis is important in ecosystem stability and productivity, we still do not have a good understanding of the link between plant diversity and fungal diversity. This is especially important in the context of conventional agricultural

practices where plant diversity is very low (i.e. crops), and leads likely to a decline of AM fungi, also because of biocides and fertilizers. Here we utilized a series of long term diversity manipulated plots to ask if decreased plant diversity leads to a decrease in fungal symbiont diversity. We analysed the AM fungal community composition and dynamics of root colonization in plots along a plant diversity gradient, using new molecular approaches involving pyrosequencing and high throughput amplicon sequence analyses (Chapter IV).

Based on this work, I suggest new ideas and prospects in terms of research and potential applications of AM fungi. I discuss the current problems of sustainable agriculture and human population increases and the question of a better use of the ecological functions of AM fungi in agriculture (Chapter V).

Chapter I:

Reciprocal Rewards Stabilize Cooperation in the Mycorrhizal Symbiosis

E. Toby Kiers,1*† Marie Duhamel,1,2 Yugandhar Beesetty,3,4 Jerry A. Mensah,4 Oscar Franken,1 Erik Verbruggen,1 Carl R. Fellbaum,4 George A. Kowalchuk,1,5 Miranda M. Hart,6 Alberto Bago,7‡ Todd M. Palmer,8 Stuart A. West,9 Philippe Vandenkoornhuyse,2 Jan Jansa,10 Heike Bücking4†

1 Institute of Ecological Science, Vrije Universiteit, 1081 HV Amsterdam, Netherlands.
2 Université de Rennes I, CNRS UMR6553 EcoBio, Campus Beaulieu, F-35042 Rennes, France.
3 Rutgers, The State University of New Jersey, Biology Department, 315 Penn Street, Camden, NJ 08102, USA.
4 Department of Biology and Microbiology, South Dakota State University, Brookings, SD 57007, USA.
5 Department of Microbial Ecology, Netherlands Institute of Ecology, Droevendaalsesteeg 10 6708 PB Wageningen, Netherlands.
6 Biology and Physical Geography, The University of British Columbia, Okanagan, Kelowna, British Columbia, Canada.
7 Estación Experimental del Zaidín (CSIC), Calle Profesor Albareda 1, Granada 18008, Spain.
8 Department of Biology, University of Florida, Gainesville, FL 32611, USA.
9 Department of Zoology, Oxford University, South Parks Road, Oxford OX1 3PS, UK. 10Institute of Agricultural Sciences, Eidgenössische Technische Hochschule (ETH) Zurich, Lindau 8315, Switzerland.
* To whom correspondence should be addressed. E-mail: toby.kiers@vu.nl
† These authors contributed equally to this work.
‡ Present address: Avenida Jacobo Camarero Sin Número, Escuela de Empresas, Nave 3, 18220 Albolote, Granada, Spain.

Published in Science 333, 880 (2011)

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 many other mutualisms, the symbiont cannot be “enslaved.” Rather, the mutualism is evolutionarily stable because control is bidirectional, and partners offering the best rate of exchange are rewarded.

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). In exchange, AM fungi provide their host plants with mineral nutrients [e.g., phosphorus (P)] and other benefits such as protection against biotic (pathogens and herbivores) and abiotic (e.g., drought) stresses (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 (Leigh, 2010; Fitter, 2006). Providing nutritional benefits can be metabolically costly, leading to the expectation that partners may defect from mutualistic duties (Kiers & van der 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 species (Vandenkoornhuyse *et al*., 2007), and fungal "individuals" can simultaneously interact with multiple host plants (Mikkelsen *et al.*, 2008) or species (Figure S1) (Selosse *et al.*, 2006). This can select for "cheaters" that exploit the benefits provided by others while avoiding the costs of supplying resources (Leigh, 2010; Douglas, 2008). It is possible that plants have evolved mechanisms to enforce cooperation by fungi, analogous to the sanctions against uncooperative partners demonstrated in diverse mutualisms (Goto *et al.*, 2010; Jandér *et al.*, 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 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 *et al.*, 1995; de Mazancourt & Schwartz, 2010). However, while mutualism market analogies have a strong theoretical

basis (Schwartz & Hoeksema, 1998; Cowden & Peterson, 2009; Hoeksema & Kummel, 2003), 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 by difficulties in manipulating the cooperative behavior of both fungal and plant partners.

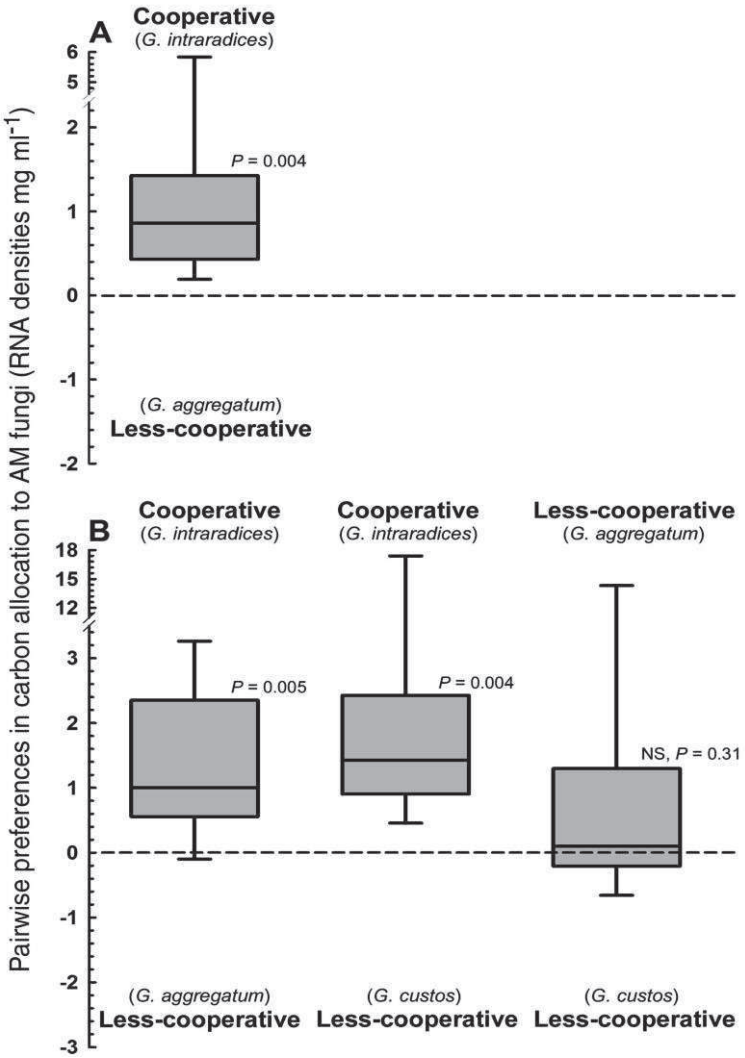


Figure 6:
Pair-wise comparisons of carbon allocation patterns to coexisting AM fungal species based on 13C enrichment. Values above the zero line indicate preferential allocation to species above the line. (A) More carbon was allocated to the cooperative species (*G. intraradices*) compared with the less-cooperative species (*G. aggregatum*) in a twospecies 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 there was 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 resolved 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 (SIP) to track and quantify plant resource allocation to individual fungal species (Figure S2) (Vandenkoornhuyse *et al*., 2007) and hence test for host discrimination against less-cooperative partners. We also employed 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.

We used the model plant *Medicago truncatula* and three arbuscular mycorrhizal fungal species within the cosmopolitan subgenus *Glomus* Ab (*Glomus intraradices*, *G. custos*, and *G. aggregatum*). These AM fungi exhibited 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 with the cooperative species (Figures S3 and 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 (Materials and methods are available as supporting material).

We grew *Medicago* hosts with one, two (*G. intraradices* versus *G. aggregatum*), or all three AM fungal species. We followed the C flux from the plant to the fungal partners by tracking plant assimilated C after 6 hours in a 13CO2 atmosphere (Vandenkoornhuyse *et al*., 2007). We harvested the roots after 6, 12, and 24 hours to follow the incorporation of host carbon into the RNA of the AM fungal assemblage. 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 on the level of 13C incorporation. By quantifying mitochondrial ribosomal RNA transcripts via specifically designed primers and quantitative polymerase chain reactions (qPCRs), we were able to track the real-time relative C allocation to each of the AM fungal species (Figures. S2, S9, and 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 13C than the RNA of both less-

cooperative species of the same genus (Figure 6). We reject the hypothesis that the less cooperative species were simply incompatible partners because colonization in all single species controls were above 80% (Figure 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 (Figure S5), suggesting either a shift in resource supply by the host to the more cooperative species or changes in competitive dynamics among the fungi (Materials and methods are available as supporting material).

The extent to which cooperation can be effectively enforced depends on the scale at which hosts discriminate against less-cooperative fungal symbionts. For plant hosts, this detection would have to occur at a very fine spatial scale (e.g., ~1 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 based on fungal signaling before colonization is unlikely because there is no reason that fungi would have to signal honestly (Leigh, 2010).

To resolve this potential paradox, we investigated 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 to track how this influences C allocation back to the fungus (Figures 7, A and B). If hosts rely on nutrient transfer as a tool to discriminate between partners on the same root (Kiers & van der Heijden, 2006; Fitter, 2006), we would predict higher C allocation to the hyphae with access to higher P resources.

We found that hosts rewarded fungal hyphae that were supplied with greater P resources. As predicted, 4 days after the addition of 14C-labeled sucrose to the root compartment, we found that significantly more C was transferred to the fungal hyphae with access to more P (Figure 7A). In the cooperative species, *G. intraradices*, even small quantities of available P (e.g., 35 *m*M) 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* (Figure 7B).

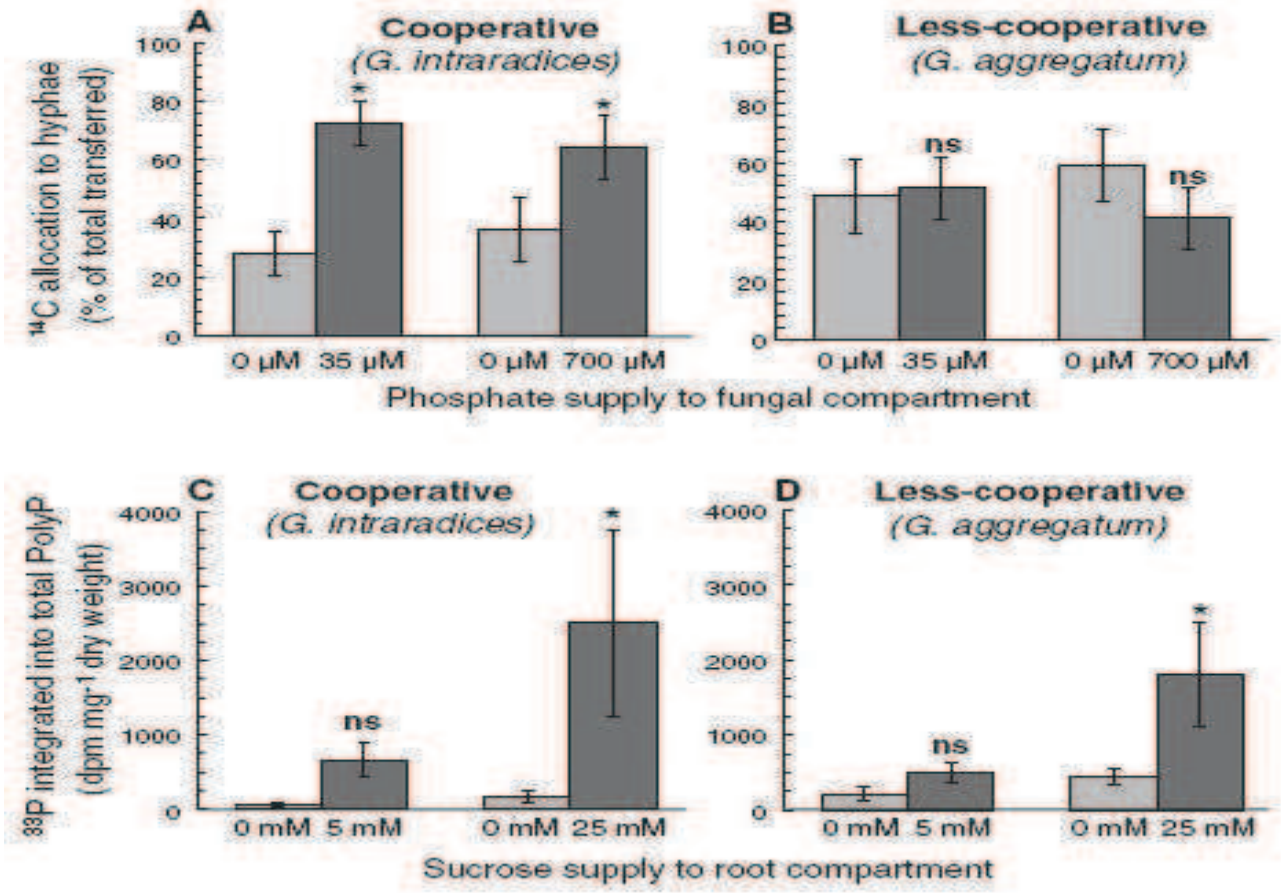


Figure 7:
Triple-plate experiments to mimic partner cooperation or defection. We found a significant effect of P availability on C allocation patterns ($F_{3,20} = 5.29$, $P = 0.0075$), with preferential allocation of C to the fungal compartments with access to more P in (A) *G. intraradices* but not in (B) *G. aggregatum*. In the reciprocal experiment, we found a significant effect of the C availability 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 (Figures S6 and S8). Asterisks indicate significant differences between treatment means (Student-Newmans-Keuls test, $P \leq 0.05$). Error bars represent the means of 8 to 10 replicates +/- 1 SEM.

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. Therefore, we used a reciprocal triple split-plate experimental design, with one fungal and two root compartments, to determine whether the fungal partner would preferentially allocate P to the host providing more carbohydrates (Figures *7*, C and D). We found that the cooperative species transferred more P to roots with greater access to C resources (Figure *7*C), confirming that fungi can discriminate among hosts differing in C supply. In contrast, the less-cooperative species, *G. aggregatum*, responded differently. Like the cooperative species, it transferred more P to the root compartment with access to more C, showing that it was able to assess and respond to the rate of C supply (Figure *7*D). However, this species predominantly stored the P resources in long-chained polyphosphates, a host-inaccessible form (Figure S6) (Takanishi *et al*., 2009). This type of resource hoarding potentially reduces P availability for competing fungi and P directly available for host uptake (Figure S8) and illustrates key differences in fungal strategies, with *G. intraradices* being a “reciprocator” and *G. aggregatum* a lesscooperative “hoarder.”

To track simultaneous resource exchange between partners, and hence determine 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-14C sucrose in either high or low concentrations, and labeled 32P 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* (Figure 8A). As above, the cooperative species responded to C rewards with a reciprocal P increase, whereas the less-cooperative species stored P in the host inaccessible formof long-chained polyphosphates (Figure S7). Finally, we compared the ratio of C costs to P transferred in both species (Figure *8*B), confirming that colonization by the less-cooperative species resulted in significantly higher host costs. These results support our whole plant SIP experiments (Figure *6*) and explain why the plant host consistently allocated more C to the cooperative species when given a choice.

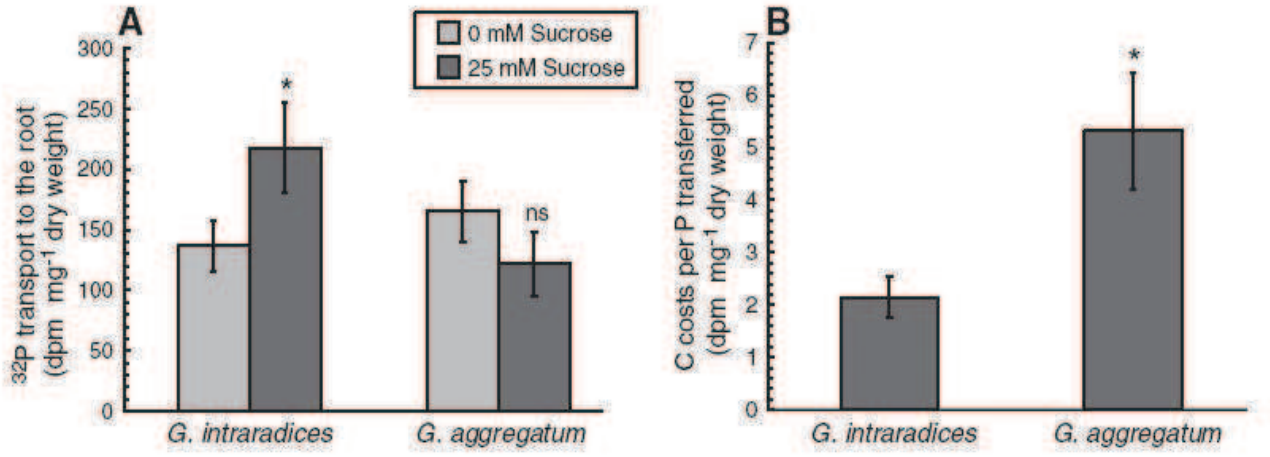


Figure 8:
Simultaneous measurement of P and C exchange. (A) Higher C availability stimulated increased P transfer by the cooperative species, *G. intraradices* (F3,22 = 3.07, P = 0.0489) but not by the less cooperative species, *G. aggregatum*. (B) When supplied with 25 mM sucrose, the carbon costs per root P of *G. aggregatum* were more than twice as high as with *G. intraradices* (F1,11 = 8.27, P = 0.0151). Dpm, disintegrations per minute. Asterisks indicate significant differences between treatment means (Student-Newman-Keuls test, P ≤ 0.05). Error bars represent means of 6 to 8 replicates +/- 1 SEM.

Overall, our results suggest that stability of the AM mutualism arises in a different way compared with 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 *et al.*, 2010), and the flowers of yucca (Pellmyr *et al.*,1994) and Glochidion plants (Goto *et al.*, 2010). In contrast, in the mycorrhizal mutualism, both sides interact withmultiple 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, nonhuman 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 *et al.*, 1995; Bshary & Noë, 2003).

Acknowledgments:

We thank R. F. Denison, J. Ellers, and N. M. van Straalen for useful discussion; Isolife, A. S. Pijl, and B. Drigo for technical assistance; B. Hause for seeds; and NSF-IOS 0943338/1051397, NSF-DEB-0827610, ANR-10-STRA-002, and the Netherlands Organisation for Scientific Research (NWO) for financial support. A.B. is one of the cofounders of Mycovitro S.L. (Granada, Spain), a company providing AM fungal inoculum for research and biotechnology purposes. Fungal cultures provided by Mycovitro are available for purchase. Plasmids containing mitochondrial DNA fragments of fungal species (provided by ETH Zurich, Switzerland) are freely available under a material transfer agreement for noncommercial use. AM fungal sequences were deposited in GenBank, accession nos. HQ706096 to HQ706103.

Supporting Material

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 (Powell *et al*., 2009; Hart & Reader, 2005; Maherali & Klironomos, 2007). (2) The fungi differentially affected growth of their host plant and this difference was evident within 10 weeks of growth (Figure S3). Although fungal benefits could potentially change (increase or decrease) over the host's ontogeny (Fitter, 1991; 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 (Cowden & Peterson, 2009). (3) The benefits conferred to hosts were consistent across different plant species (Figure S3). This allowed us to reject the hypothesis that the observed differences in mutualistic benefit were attributed to local coevolutionary 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.*, 2009, Husband *et al.*, 2002; Smith *et al.*, 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 (Figure 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 (Husband *et al.*, 2002). (Johnson *et al.,*1997) 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 (Figure 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, 1993; Johnson *et al.*, 1992). (Kiers & van der Heijden, 2006) Importantly, we do not categorize our less-cooperative species as unequivocal 'cheats' (Smith *et al.*, 2003; Smith *et al.*, 2011; Douglas, 2010). 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). (Fitter, 2006) 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 (Arnaud *et al.*, 1996) and with sucrose as the only carbon source. We 3 ultured 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, i.e. 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 nonmycorrhizal 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) (Figure 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 (Figure S10). The detection limits were calculated from the background region of the qPCR response curve as follows: where DL represents the detection limit of the assay (Cq value), AVCq(back) the mean of the Cq values in the background region and SD (AVCq(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 H2SO4 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. 5 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 13CO2 labeling. 13CO2 labeling and harvest Plants were labeled with 13CO2 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 12CO2 from a pressurized cylinder. During the night period prior to labeling, 12CO2 was removed by a CO2-scrubber in accordance with the 12CO2-respiration of *M. truncatula*. One hour before the start of the day period, 13CO2 was injected from a pressurized cylinder (99 atom % 13C, 1 atom % 12C; Isotec, Inc. Miamisburg, OH, USA). For 6 h, a total CO2-level (12CO2 + 13CO2) of 396±20 µl l-1 CO2 was maintained. The 13C-enrichment of the atmosphere was 92% at the start of the 6-h labeling period. This value gradually decreased due to the 12CO2 respiration by the plant and resulted in a mean 13C-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 13CO2. After the flushing period, the labeling chamber was closed and the 12CO2 level was maintained at 405±29 µl l-1. To follow the incorporation of 13C 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 N2. 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 691,1128 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 (Drigo *et al.*, 2010). 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 13C 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 (Figure 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: where a and b are constants, x0 is the x value of function peak, and e is the base of natural logarithm (approximately 2.718). Only the samples with R2 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 Figure S2 for example) mean higher 13C 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 13C 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 (Figure *6*) 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 13C 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 lesscooperative 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 (Figure 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*, Figure 8A), and represented the maximum and minimum of the host benefit continuum (Kiers & van der Heijden, 2006; 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 2002; Bago *et al.*, 2003; Olsson & Johnson, 2005; Olsson *et al.*, 2005; Bücking & Shachar-Hill, 2005; Jin *et al*., 2005; Govindarajulu *et al*., 2005; Hammer *et al*., 2011; Tian *et al.*, 2010). 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 can not 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 (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 (KH2PO4 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 (Figures 7A,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 KH2PO4) 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 (Figures 7C,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 KH2PO4 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 (Figure 8A) 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 (Figure 8B)? To achieve both these aims, we used a two compartment Petri dish system with one root and one fungal compartment to which simultaneously 14C–sucrose and 32P-orthophosphate were added. Three weeks after the ERM started to cross over the divider, we added [U-14C] sucrose with a specific activity of 498 mCi mM-1 (Sigma-Aldrich, St. Louis, USA) to the root compartment and [32P] 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 14C labeled sucrose and 14C labeled sucrose diluted with non-labeled sucrose for a final sucrose concentration of 25 mM sucrose (0.448 µM as 14C 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 14C and 32P according to the emission energy in dual isotope experiments. The 14C 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 32P passed), when 32P 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 (Figures 7C,D, Figures 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 BaCl2 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 33P content in all fractions was determined by liquid scintillation counting.

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 ($P \leq 0.05$).

Supplementary 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

eachother. Various mechanisms have been proposed to explain, for example, how mycorrhization may be mediated by host plants (Bonfante & Genre, 2010; Douglas, 2008; Blee & Anderson, 1998; Pearson *et al.*, 1993; Schaarschmidt *et al.*, 2007; Vierheilig, 2004). 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 *et al.*, 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 (Atul-Nayyar *et al.*, 2009; Tanaka & Yano, 2005; 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 (Kobae & Hata, 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.

Supplementary Figures S1-S10

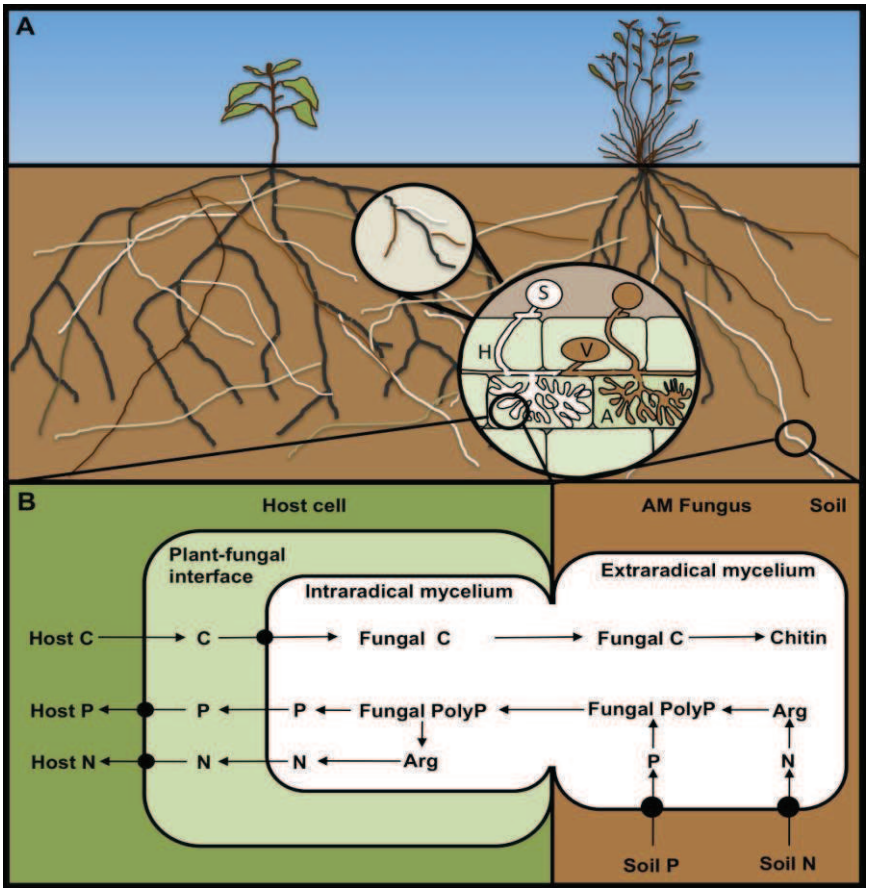


Figure S1:

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 (Pi) and Arg. Arg is further broken down to inorganic N (specifically NH4 +), and then transferred across the plant-fungal interface.

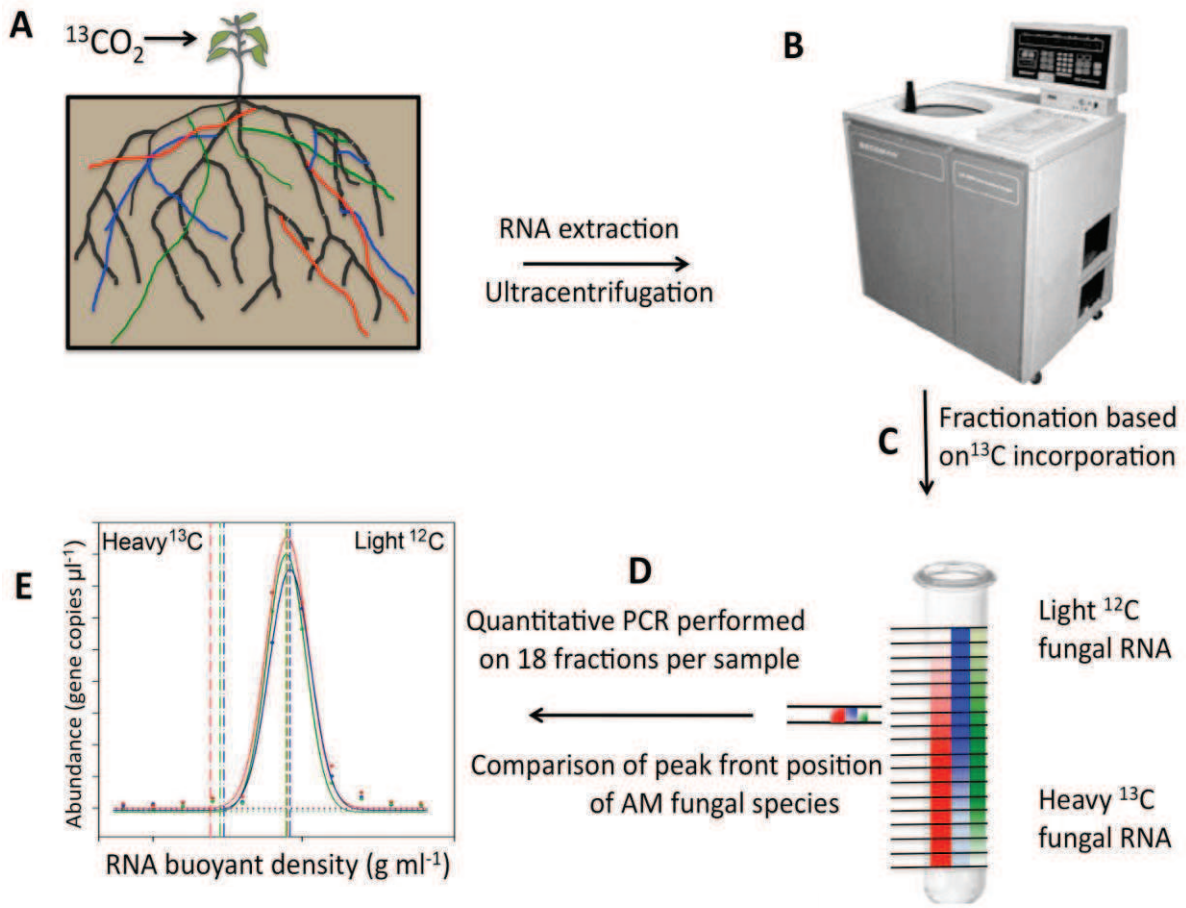


Figure S2:

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 13CO2 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 13C-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 13C 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.

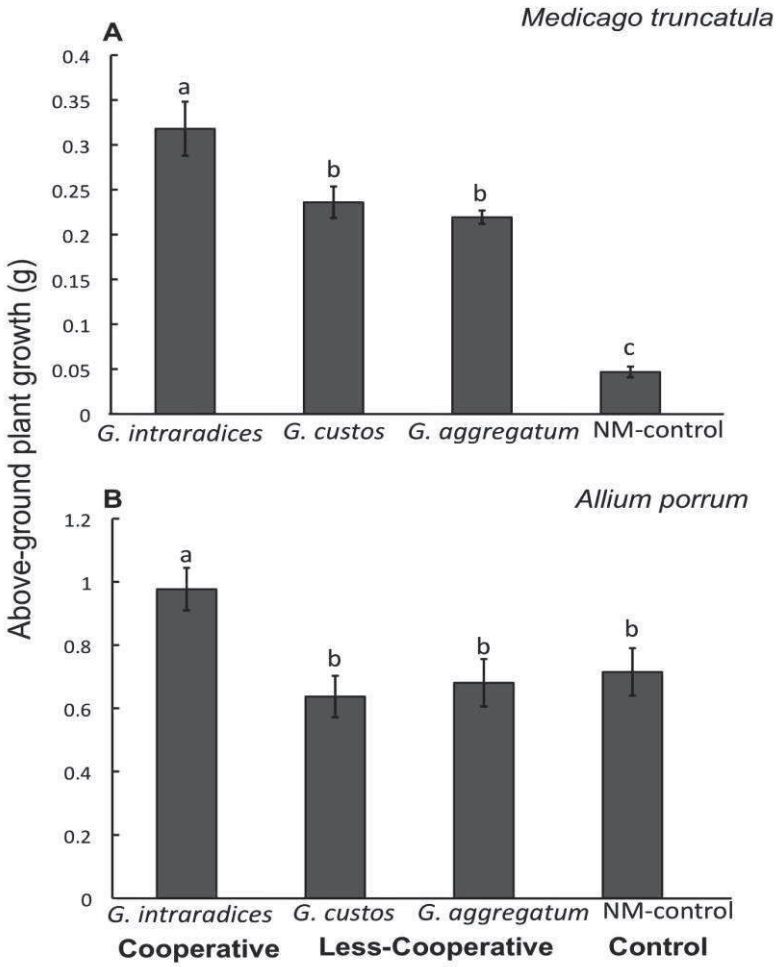


Figure S3:

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, $P \leq 0.05$). 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 andrespond 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.

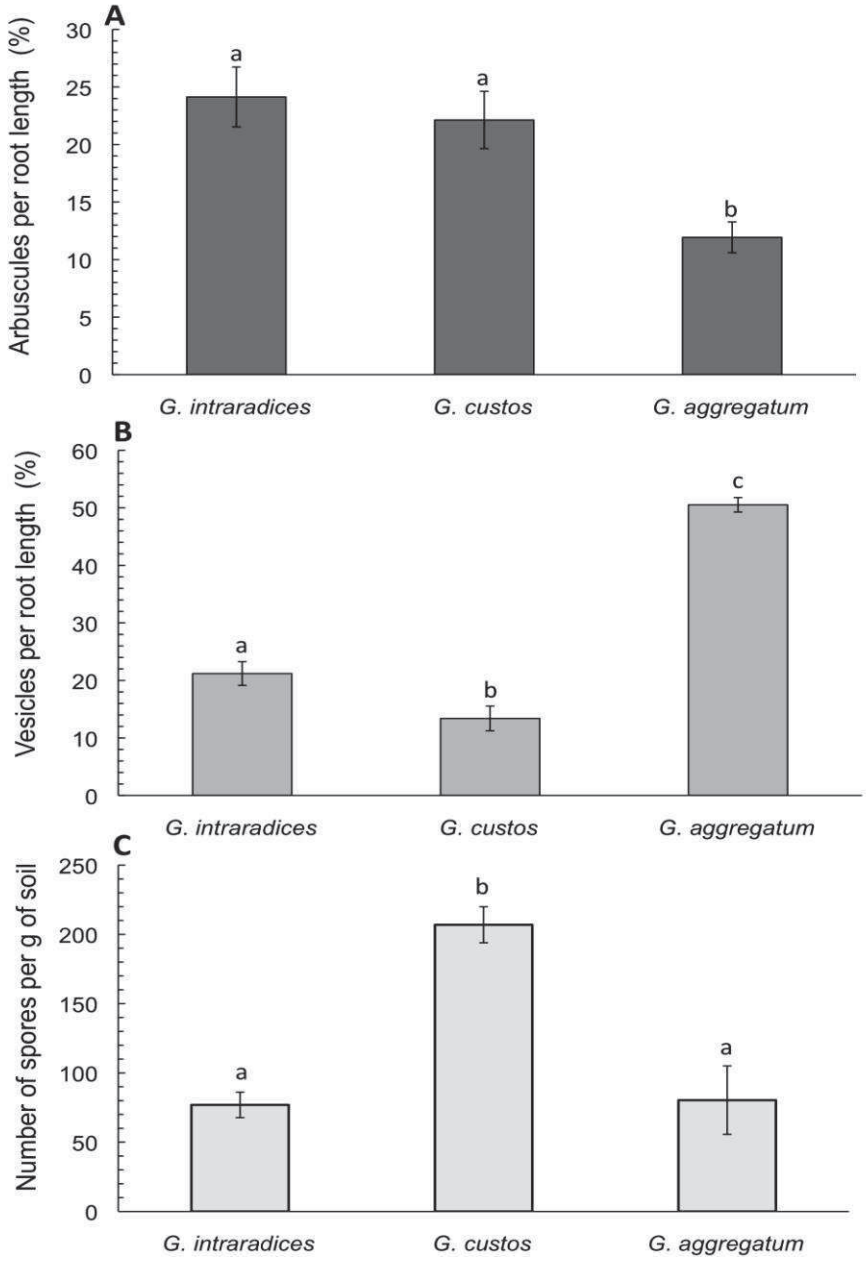


Figure S4:

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.

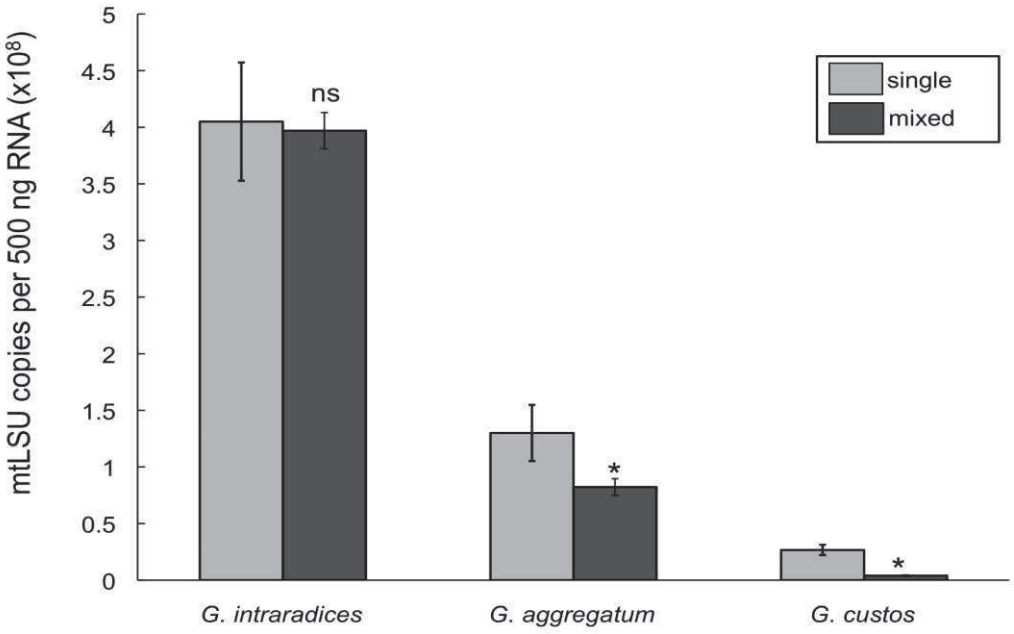


Figure S5:

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.

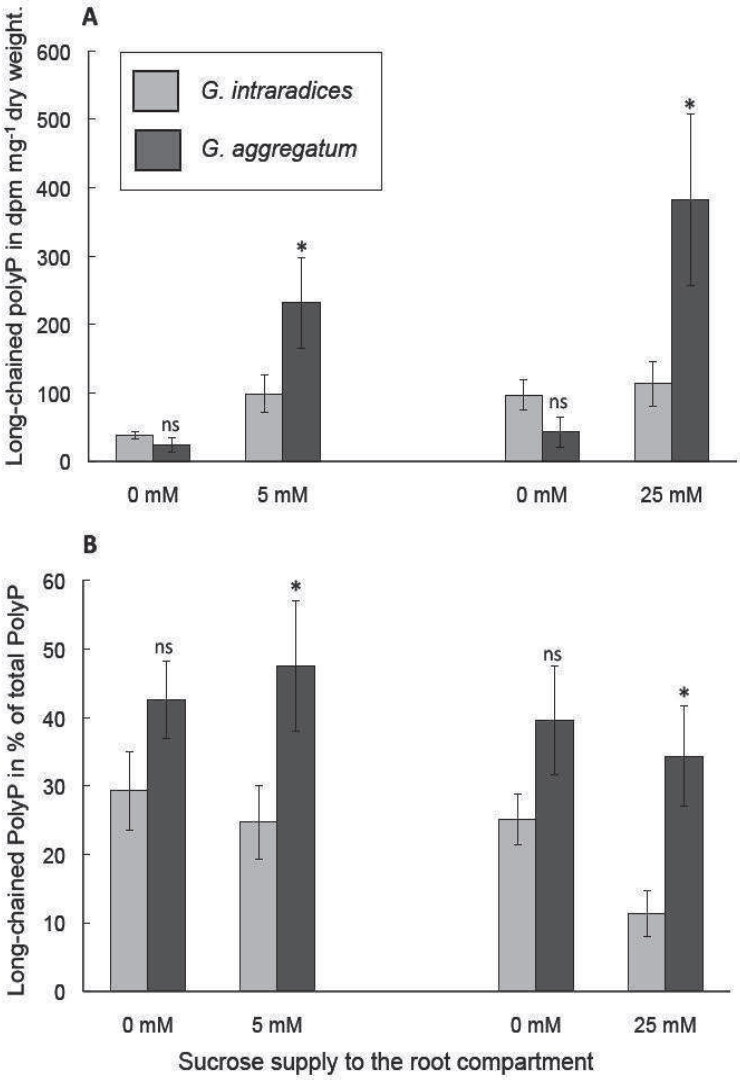


Figure S6:

Long-chain PolyP pools of cooperative and less-cooperative AM fungi in a onefungal, 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 Figures S7, S8). In contrast, the cooperative fungus *G. intraradices* converted a larger proportion of its long-chained PolyP to shortchained 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 (Takanishi *et al.,* 2009; Ohtomo & Saito, 2005). Longchained PolyP concentrations were higher in roots that were colonized with the lesscooperative 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.

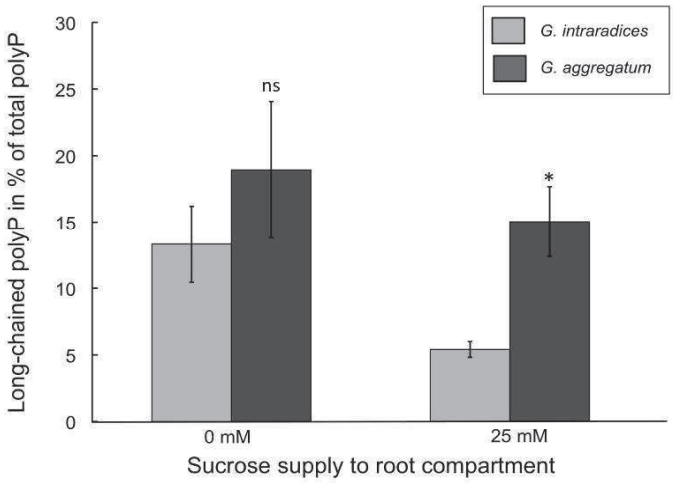


Figure S7:

The less-cooperative *G. aggregatum* retained significantly more P in form of longchained polyphosphates (PolyP), than the cooperative AM fungus *G. intraradices*. As in the triple-plate experiement (Figure 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.

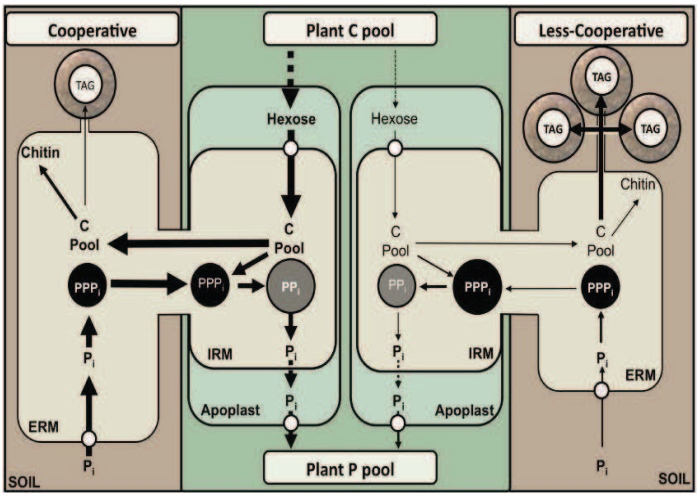


Figure S8:

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 (Figure 6), 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) (Figures 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 (Takanishi *et al.,* 2009; Ohtomo & Saito, 2005).

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 (Javot *et al.*, 2007; Harrison *et al.*, 2002). 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 (Figure S4), and less into the development of nutrient absorbing ERM. Phosphate that is transferred to the IRM ofthe less-cooperative fungus is stored mainly in the form of long-chained polyP, and conversion to short-chained polyP is low (Figures 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 (Bücking & Shachar-Hill, 2005, Smith *et al.,* 1994a; Smith *et al.,* 1994b; Ferrol *et al.*, 2002). 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 *et al.*, 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 (Figure *8*). 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.

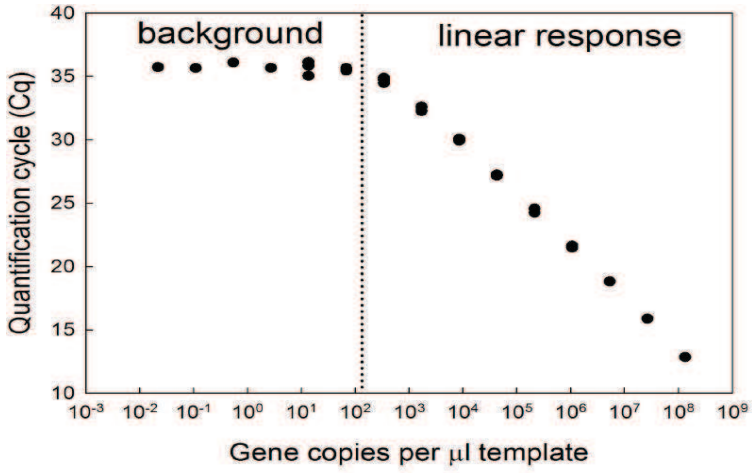


Figure S9.

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.

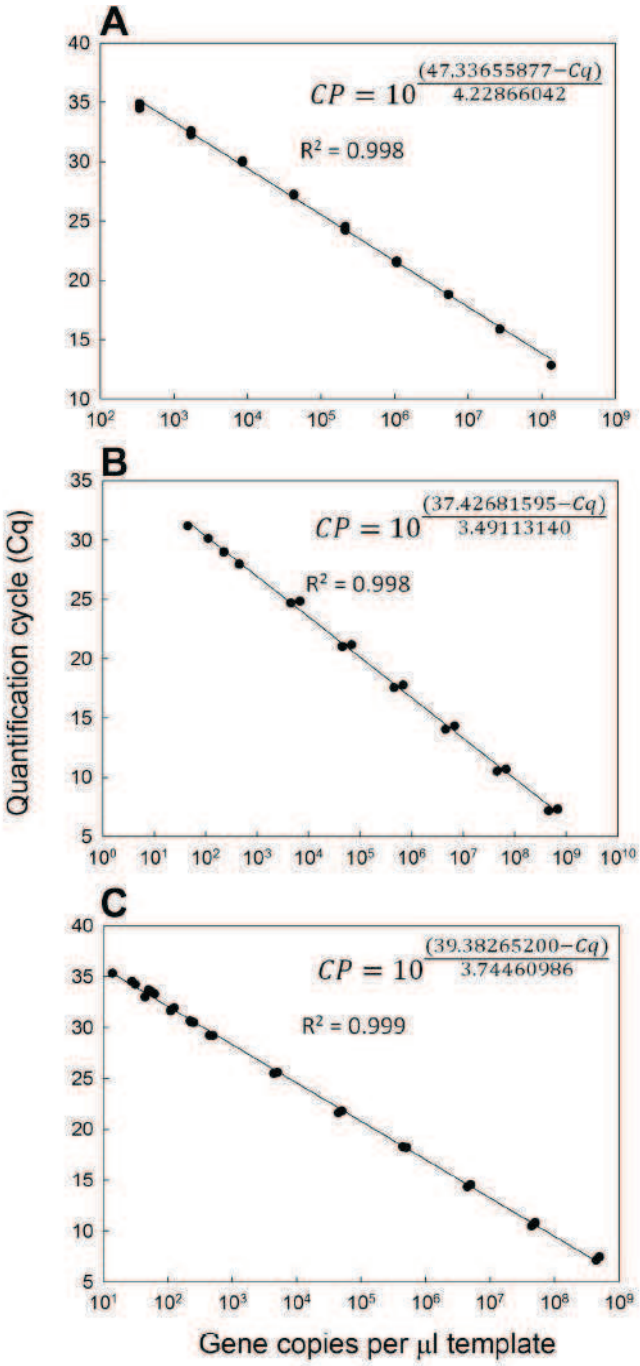


Figure S10:

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.

Supplementary Tables S1-S3

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
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 assays.

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

Chapter II:

Do fungivores trigger the transfer of protective metabolites from host plants to arbuscular mycorrhizal hyphae?

Marie Duhamel1,2,5 †, Roel Pel2†, Astra Ooms2, Heike Bücking3, Jan Jansa4, Jacintha Ellers2, Nico M. van Straalen2, Tjalf Wouda2, Philippe Vandenkoornhuyse1, E. Toby Kiers2

1Université de Rennes I, CNRS UMR6553 EcoBio, Campus Beaulieu, F-35042 Rennes, France.

2Institute of Ecological Science, Vrije Universiteit, 1081 HV Amsterdam, Netherlands.

3Department of Biology and Microbiology, South Dakota State University, Brookings, SD 57007, USA.

4Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14220 Praha 4, Czech Republic.

† These authors contributed equally to this work.

Accepted in Ecology (2013)

Abstract

A key objective in ecology is to understand how cooperative strategies evolve and are maintained in species networks. Here, we focus on the tri-trophic relationship between arbuscular mycorrhizal (AM) fungi, host plants and fungivores to ask if host plants are able to protect their mutualistic mycorrhizal partners from grazing. Specifically, we test whether secondary metabolites are transferred from hosts to fungal partners to increase their defence against fungivores. We grew *Plantago* lanceolata hosts with and without mycorrhizal inoculum, and in the presence or absence of fungivorous springtails. We then measured fungivore effects on host biomass and mycorrhizal abundance (using quantitative PCR) in roots and soil. We used high-performance liquid chromatography to measure host metabolites in roots, shoots and hyphae, focusing on catalpol, aucubin and verbascoside. Our most striking result was that the metabolite catalpol was consistently found in AM fungal hyphae in host plants exposed to fungivores. When fungivores were absent, catalpol was undetectable in hyphae. Our results highlight the potential for plant-mediated protection of the mycorrhizal hyphal network.

Key words: cooperation, defense, mutualism, networks, species interactions, symbiosis.

Introduction

All mutualistic interactions are embedded in larger ecological webs (Bascompte, 2009). This means that external species, including predators, parasites, herbivores, and even other mutualists (e.g. Palmer *et al.,* 2010) can influence the benefit:cost ratios of mutualisms, and alter theirecological and evolutionary outcomes (Afkhami & Rudgers, 2009). Anthropogenic disturbances are increasingly linked to the disruption of species networks (Kiers *et al.*, 2010), and this has prompted a call to focus on understanding how cooperative strategies evolve and are maintained in species networks (Bascompte, 2009). The 450-million-year-old arbuscular mycorrhizal (AM) symbiosis is likely the world's most prevalent mutualism (van der Heiden *et al.*, 2008). It primarily involves the exchange of carbohydrates from plants for mineral nutrients from the fungal partner (Parniske, 2008). Estimates suggest that up to 20% of total host carbon can be transferred to AM fungi (for review see Bago *et al.*, 2000). In return, AM fungi improve the host plant's supply of phosphorus (Parniske, 2008), and nitrogen (Fellbaum *et al.*, 2012) and provide a diversity of other benefits to the host plant (van der Heiden *et al.,* 2008). The symbiosis contributes to massive global nutrient transfer, global carbon sequestration, and soil stabilization (Rilling & Mummey, 2006). These features make it paramount to health and ecosystem function. Like all mutualisms, the mycorrhizal symbiosis exists in a rich web of interactions. A given host is colonized by multiple AM fungal species (e.g. Vandenkoornhuyse *et al.*, 2002), and a single fungus can simultaneously colonize several plant individuals belonging to different plant species (e.g. Vandenkoornhuyse *et al.*, 2007, Mikkelsen *et al.*, 2008). This common mycelial network represents a dynamic underground environment: AM fungal hyphae can account for up to 30% of the total soil microbial biomass (for review see Leake *et al.*, 2004).

The plant-AM fungal network co-exists with populations of soil micro-arthropods (Hishi *et al.*, 2008) that feed on rhizosphere fungi, including AM fungal hyphae (Jonas *et al.*, 2007). Collembola, known collectively as springtails, are among the most abundant soil arthropods (Petersen & Luxton, 1982) and most Collembola species feed on fungal hyphae (Fountain & Hopkin, 2005). Depending on their densities, fungivores may either enhance or degrade the symbiosis (Gange, 2000). At low densities, the presence of fungivores has been shown to increase AM fungal colonisation and hyphal development by acting as a transporting agent for nutrients in the soil (Klironomos & Moutoglis, 1999;

Bakonyi *et al.*, 2002). Conversely, when fungivore densities increase, grazing pressure can negatively affect the AM fungal hyphal development (Klironomos & Ursic, 1998). This grazing effect can represent a significant cost to the AM fungi and their host plants (Harris & Boerner, 1990; Klironomos & Ursic, 1998; Johnson *et al.*, 2005).

It is well known that plants employ a great variety of biologically active secondary metabolites as defensive compounds to deter herbivores (Bowers & Puttick, 1988; Marak *et al.*, 2002; Wurst *et al.*, 2010), but it is unknown if soil fungi use a similar chemical-based defense strategy. Recent work suggests that soil-borne fungi have developed strategies to decrease their palatability (Böllmann *et al.*, 2010; Kempken & Rohlfs, 2010), such as the utilization of poisonous or repellent compounds to discourage hyphal consumption. So far only a few examples of fungal-synthesized repellants have been described (Rohlfs *et al.*, 2007; Böllmann *et al.*, 2010; Staaden *et al.*, 2010). Relative to saprotrophic free living fungi, mycorrhizal fungi (both AM and ectomycorrhizal types) appear to be well protected from grazing by fungivores. Several laboratory-based food choice studies have shown that fungivorous springtails preferentially consume saprophytic free-living fungi over mycorrhizal taxa (e.g. Klironomos & Kendrick, 1996; Klironomos & Ursic 1998; Schreiner & Bethlenfalvay, 2003). When AM fungal hyphae are the only available food source, a diminished growth performance and fecundity is found in many springtail species (Klironomos & Moutoglis, 1999; Larsen *et al.*, 2008), suggesting that the consumption of these hyphae may be disadvantageous (Gange, 2000; Kempken & Rohlfs, 2010; Böllman *et al.*, 2010). There is also some evidence that plant colonization by AM fungi can induce protective secondary metabolites in roots and leaves (Gange & West, 1994; De Deyn *et al.*, 2009). The question arises, whether fungal partners benefit, either directly or indirectly, from secondary metabolites production of their plant host.

Here we test the idea that secondary metabolites, used by the host plant for its own protection against herbivory, can be transferred to the fungal partner to increase its defense against fungivores. We hypothesize that the presence of fungivores elicits the transfer of secondary metabolites to the fungal hyphae by the mycorrhizal plant. To test this hypothesis, we utilized microcosms to study the interaction between the host plant *Plantago* lanceolata, *Glomus* sp. fungal symbionts, and the fungivorous springtail *Folsomia* candida. We focused on the production of catalpol, aucubin and verbascoside, the main defensive secondary metabolites known to occur in *P. lanceolata* (Bowers *et al.* 1992). Catalpol and aucubin are iridoid glycosides and act as direct defense compounds

(Fontana *et al.*, 2009), with generalist anti-feedant properties (Bowers & Puttick, 1988; Biere *et al.*, 2004), and antimicrobial activity (Marak *et al.*, 2002). Verbascoside is a caffeoyl phenylethanoid glycoside known for its antimicrobial and cytotoxic activity (Pardo *et al.*, 1993). We used high-performance liquid chromatography (HPLC) to measure secondary metabolite concentrations in roots, shoots and fungal hyphae and quantitative PCR (qPCR) to determine the mycorrhizal abundance in roots and soil in the presence and absence of fungivores. Ultimately, our aim was to determine if plants protect their mycorrhizal hyphae in the presence of fungivores.

Methods

P. lanceolata was chosen as the host plant because it has become a model plant species in mycorrhizal research: it is readily colonized and highly responsive to a broad range of AM fungal taxa (Maherali & Klironomos, 2007; Verbruggen *et al.* 2012) and is known to employ secondary metabolites for defense and protection (Marak *et al.*, 2000; Biere *et al.*, 2004; De Deyn *et al.*, 2009; Wurst *et al.*, 2008, 2010). *P. lanceolata* seeds (Cruydthoek, Assen, the Netherlands) were sterilized using diluted bleach (NaOCl 2.5 % w/w), then planted in autoclaved quartz sand (15% humidity) and grown for 14 days under plastic foils.

For each pot, three randomly selected seedlings of *P. lanceolata* (three seedlings to ensure ample root growth and hyphal growth) were planted together in a single meshbag (Ø 6,5 cm, height 16 cm). The mesh bags were prepared from 20 µm pore size nylon mesh, which allowed the hyphae, but not the roots, to cross over and protected the roots from springtail exposure (Appendix A). After filling the quartz-dune sand mixture in the bags, they were placed central in pots (Ø=15 cm) with autoclaved quartz sand (~15% humidity, 1.7 kg per pot) mixed with 25% (w:w) glass beads (4 mm in diameter) to create spaces for springtails.

Seedlings were inoculated with spore material produced in in vitro root organ cultures (provided 140by Mycovitro S.L. Biotechnología ecológica, Granada, Spain) in one of three treatments. The seedlings were inoculated either with: (1) a single fungal inoculum of *G. intraradices*, strain 09 (Schenck & Smith, 1985, see Stockinger *et al.*, 2009 for discussion of *G. intraradices* re-classification), (2) a single fungal inoculum of *G. custos*,

strain 010 (Cano *et al.*, 2009) or (3) a 50:50 mixture of *G. intraradices* and *G. custos*, (see Kiers *et al.*, 2011 for further description of fungal species). In all cases, a total of ~1000 spores were added to the roots of the host plants in each mesh bag. Non-mycorrhizal controls were inoculated with heat-sterilized inoculum. Pots were randomized into treatments with or without springtails of ten replicates each.

We used the springtail *Folsomia* candida (Berlin clonal line), a ubiquitous soil microarthropod with a global distribution (Fountain & Hopkin, 2005) as our fungivore. This Collembolan has been shown to consume AM fungal hyphae, although saprophytic fungi are the preferred food source (Gange, 2000; Larsen *et al.*, 2008). Individuals of *F. candida* (size range 0.25 to 0.5 mm) were raised in a climate room at 15°C, fed with a diet of common baker's yeast, and starved for a week before being added to pots. One month after transplanting seedlings, we added 200 *F. candida* per pot, outside the meshbag in a shallow trench, providing a final density of approximately 120 individuals kg-1 soil (i.e. 1.4 x 10^4 individuals / m2). Our aim was to match Collembola density found in natural habitats, which vary in grasslands from 0.5 to 8 x 10^4 individuals / m2 (Petersen & Luxton, 1982) to agricultural fields with densities from 0.5 to 2.5 x 10^4 (Moore *et al.*, 1984). The plants were grown for twelve weeks in a greenhouse (temperature 16020-25 °C, relative humidity 60-70%), and watered to maintain ~15% humidity. A Hoaglands nutrient solution with a reduced P content (50%) of 4 ml kg-1 dry sand was added once every two weeks (Hoagland & Arnon, 1950, see also Appendix A). Pots were randomized on benches once per week.

Harvest

At harvest, the aerial plant portions were removed, freeze-dried and weighed. The roots were removed from meshbags, washed, freeze-dried, weighed and a subsample was taken for DNA extraction. Both roots and shoots weights were corrected for raw ash content. One soil core (diameter 2.7 cm) was collected outside the meshbags and weighed for DNA extraction and qPCR. Glass beads were removed from the soil and cores were stored at -20°C until DNA extractions. To measure the hyphal mass, blocks of sand were removed from the pot, placed on a sieve with a 0.5 mm mesh and subjected to wet sieving/washing (Appendix A). The ERM fraction was snap-frozen in liquid N2, freeze-dried, weighed, and stored at -80°C for later HPLC-analysis. Fungal biomass was determined as ash-free dry weight by the weight difference upon loss on ignition at 500°C (see below, Chemical analysis). A random subsample of roots were stained using the

modified method by Phillips and Hayman (1970) with Trypan Blue in lactoglycerol, following maceration of the roots with KOH (10% for 30 min at 90°C) and acidification with 1% HCl for 15 min. The roots were then aligned on a slide and 100 intersections were scored for presence/absence of hyphae, arbuscules and vesicles using the method described by McGonigle *et al.* (1990).

Molecular analysis

We extracted fungal DNA from roots using DNeasy Plant Mini Kit (Qiagen) and soil using FastDNA SPIN kit for Soil (MP Biomedicals) following manufacturer´s recommendations. One gram of crushed (with vortexer), thawed (but not dried) soil was used for the extraction. For root extractions, fresh-roots were blotted dry, cut into small pieces and mixed, with a random subsample of 100 mg fresh weight taken for further processing. Liquid nitrogen and micropestles were used to pulverize the roots, following supplier recommendations, with DNA eluted in 50 µl elution buffer. The abundance of the two AM fungal species in the different samples was quantified using taxon-specific markers with hydrolysis probes (Kiers *et al.,* 2011; Thonar *et al.,* 2012, Appendix B). Our qPCR tests revealed that the inoculation with the AM fungal species *G. custos* was unsuccessful. This fungal species was undetectable in qPCR tests in most root samples that were inoculated with this strain (both single and mixed), and we found no hyphal biomass in the single *G. custos* treatment. In only one out of four analyzed plants that were inoculated with *G. custos*, a positive qPCR was recorded, but the abundance was still 2 orders of magnitude lower than for *G. intraradices*. We therefore removed the treatments containing *G. custos* from our plant analyses. However, we did still test for the presence of secondary metabolites in the hyphae of the mixed (*G. intraradices* + *G. custos*) treatment. Although this treatment only contained *G. intraradices* (i.e. *G. custos* did not successfully colonize hosts), it was still a valid test for the presence/absence of secondary metabolites in AM fungal hyphae.

Chemical analysis

Freeze-dried roots and shoots were ground to powder using a metal lockable tube and a metal bullet for 50 seconds at the highest speed (30 strokes sec-1, Retsch MM200). The hyphae were ground cryogenically at liquid N2 temperature in an eppendorf tube

using a fitting pestle. The powdered roots, shoots and hyphae were stored at -80°C until HPLC analyses. The secondary metabolites were extracted from 10 mg of leaf or root material in 2 ml of methanol, following a modified analytical protocol used by Sesterhenn *et al.* (2007) for iridoid glycoside determination. The extraction vials were sonicated for 4 min, heated for 30 min at 50°C and shaken overnight at 150 rpm. Subsequently, samples were centrifuged at 3000 rpm for 10 minutes, and filtered through 4.0 µm. Because no suitable internal standard was available, care was taken at all steps to maintain the absolute (secondary metabolites) concentration of the methanol extracts. For HPLC analysis, 50 µl of the extract and 100 µl of mobile phase A were transferred to a vial (Appendix A). The preparation of the hyphal extracts followed the same protocol as the plants, except that 10 mg of crude hyphal material was extracted in 1.5 ml methanol. The concentrations of hyphal extracts were then increased 10-fold by evaporation of the methanol under a stream of N2. For hyphal measurements, there was a total of 6 replicates in the *G. intraradices* + springtail treatment, and 7 replicates in the mixed AM fungi + springtail treatment because some samples were pooled to achieve a sufficient amount of hyphae. All freeze-dried hyphal material was cryogenically ground and a subsample was subsequently ashed at 500°C to measure fungal biomass (the ash-free dry weight) by the loss on ignition.

Hyphal isolation from in vitro cultures

To begin to assess if secondary metabolites originated from the fungus itself, we analysed secondary metabolites in hyphae from in vitro root organ cultures (Doner and Bécard, 1991). In vitro grown hyphae from *G. intraradices* (provided by Mycovitro) were grown on a gellan gum medium on a split plate together with carrot roots (Daucus carota). To extract the hyphae from 2the medium, the fungal compartment of the medium was suspended in 25 ml 10 mM sodium citrate buffer (pH 6, 37 °C, Appendix A). The hyphae were freeze-dried, stored and processed as above.

Choice tests in presence and absence of catalpol

To determine if catalpol was a feeding deterrent for *F. candida*, we constructed food choice arenas as described by Larsen *et al.* (2008). We divided Petri dishes with plaster of Paris bottoms into two equal sections using a transverse wall, while leaving an opening to

allow migration of springtails to either section. One section of the arena received clean yeast, the other side yeast with the catalpol spiked at 4 different treatment concentrations: 0, 0.1, 1,0 or 2.0% w/w. We used yeast rather than fungal mycelium because: (i) a high amount was required for all the different choice treatments, (ii) it is a more uniform test material than mycelium grown on a series of replicate plates, and (iii) it is free of any possible secondary metabolite material. We placed 20 springtails in the middle opening of each arena with 10 replicates per treatment. We recorded the distribution of the springtails over the two sections 4 times per day for 3 consecutive days. For each arena the collembolan distribution was averaged per day, and each treatment was tested for significant deviations from a random 50:50 distribution. The first day was not taken into account since the springtails were still actively exploring both sections.

Statistical analysis

Plant data, hyphal biomass and secondary metabolite concentrations were analyzed using a two-way ANOVA with R 2.13.0 (http://www.r-project.org/). If significant differences were found with ANOVA, a Tukey post-hoc test was applied. All data were first tested for normality and homogeneity of variances (Kolmogorov-Smirnov test & Levene's test) and a logarithmic link function was used when required. To confirm ANOVA test results, a complementary statistical analysis was performed using parametric generalized linear model (GLM), using R (GLM approach, Appendix A). All molecular analyses and data on secondary metabolites in hyphae were analyzed by a one-sample t-test. Differences were considered significant at $p<0.05$. We ran a power analysis using R 2.13.0 (http://www.r-project.org/) on hyphal biomass and the molecular root colonization data to determine the number of replicates we would have needed to detect a significant difference with a given power of 90% possibility to detect a significant result with $p<0.05$.

Results

To investigate the potential transfer of protective secondary metabolites from the host plant to the fungal symbiont, we studied the effects of AM fungi on *P. lanceolata* in the presence or absence of springtails (Overview table and statistics, Appendix C and D). We found a highly significant effect of AM fungal inoculation (ANOVA: df=38, F=50.2, $p<0.001$)

and springtail treatment (ANOVA: df=38, F=17.8, p<0.001) on aboveground plant growth (Figure 9). The inoculation with *G. intraradices* led to an increase in aboveground growth of 74% and of 60% , respectively in the presence (Tukey, p<0.05) and in the absence of springtails (Tukey, p<0.001). We also found that the presence of springtails reduced the positive effect of AM fungi on plant biomass (23% vs 60%, Figure 9), as indicated by a significant interaction term (ANOVA: df=38, F= 4.12, p=0.0498). There was no significant effect of inoculation or springtail addition on root biomass (ANOVA: df=36, F=1.27, p=0.299, Figure 9). As discussed above, treatments containing *G. custos* were excluded from plant growth analyses because of a failure to successfully establish *G. custos* colonization.

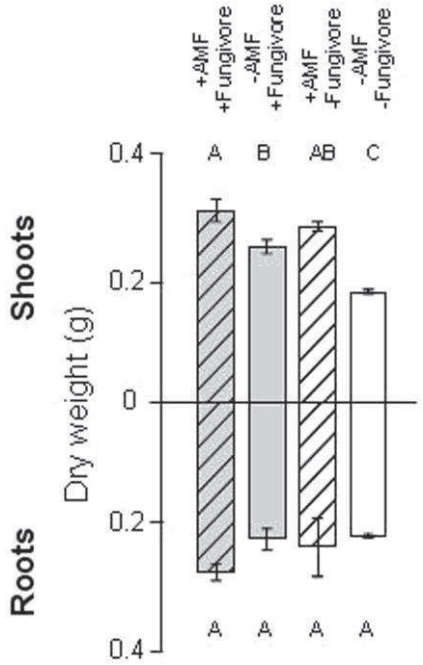


Figure 9:
Effects of AM fungal species *G. intraradices* (hatched bars) and the fungivore *F. candida* (grey bars) on *P. lanceolata* aboveground and belowground biomass. Above the x-axis corresponds to shoot dry weight, below the x-axis corresponds to root dry weight. Letters indicate significant differences between treatments means according to Tukey's HSD test (P ≤ 731 Bars represent the means of 10 replicates ± 1 standard error.

We measured AM fungal colonization in both the soil outside the meshbags (using hyphal biomass corrected for raw ash content as well as qPCR) and roots (using qPCR and visual counts) of the *G. intraradices* microcosms (Figure 10). In the soil, hyphae of *G. intraradices* were found (biomass and by qPCR) in all but one pot. We found no significant reduction in the hyphal biomass in the soil in the presence of springtails (Student's t-test, t = 0.93, df = 18, p>0.05, Figure 10a). Based on our microscopic counts, we found the plant roots had a total mean colonization of 57%, with no significant effect from the springtail treatment (ANOVA, f = 0.6434, df = 1, p>0.05), nor in % vesicles (Mean = 12%; ANOVA, f = 0.6933, df = 1, p>0.05) or arbuscles (Mean = 46%; ANOVA, f = 0.538, df = 1, p>0.05).

Likewise, in the qPCR analysis, we found no statistical difference on soil fungal colonization due to springtails (Student's t-test: t = 0.3025, df = 12, p>0.05, Figure 10b), nor on root colonization (Student's t-test: t = 0.36, df = 12, p>0.05, Figure 10c). To determine if these non-significant differences were due to the low sample number, we ran a power analysis and found that between 17, ~100, ~1000 samples would be needed to detect differences in springtail addition on root biomass, hyphal biomass and qPCR data.

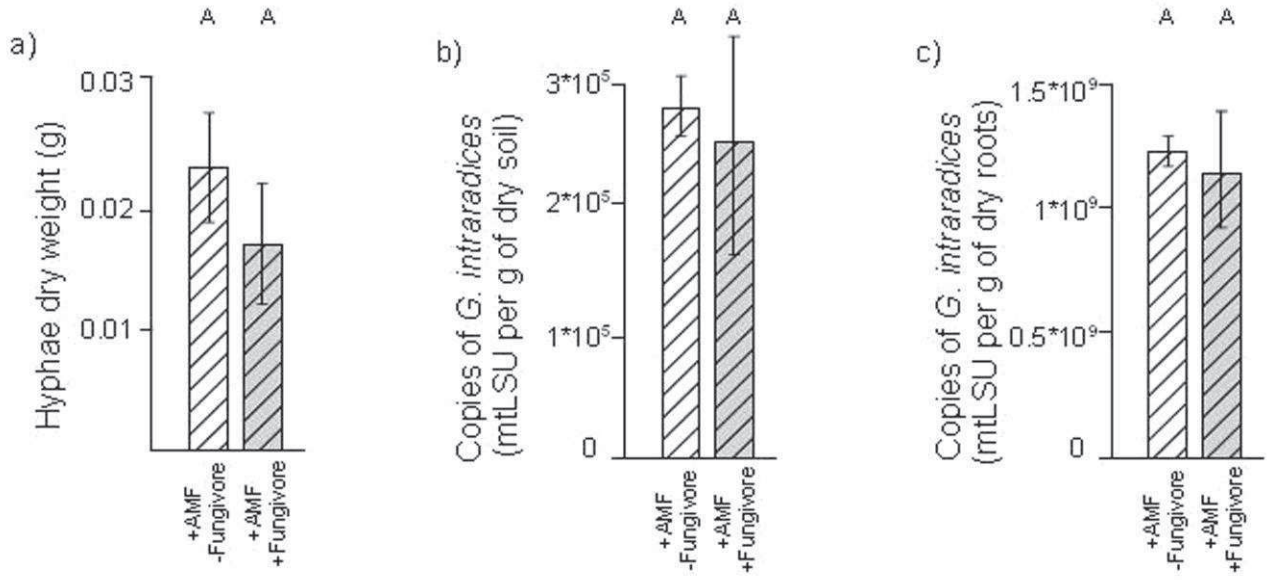


Figure 10:

Effect of F.candida presence (grey) and absence (white) on (a) the mean dry weight of *G. intraradices* hyphae, N=10, (b) mtLSU copies of *G. intraradices* per gram of soil, and (c) mtLSU copies of *G. intraradices* per gram root, N=7, ± 1 standard error. Letters indicate significant differences between treatments according to Tukey's HSD test (P ≤ 0.05).

P. lanceolata roots and shoots contained the secondary metabolites catalpol, aucubin and verbascoside in varying concentrations depending on the treatment. Fungal inoculation led to a decrease in the verbascoside root concentration of 62.5% in the absence of springtails (ANOVA: df=36, F=7.62, p<0.001, Figure 11a). This trend was reversed in shoots where AM fungal inoculation increased the verbascoside concentration by 37%, but only when springtails were present (ANOVA: df=36, F=4.41, p=0.001, Figure 11a). In contrast, AM fungal inoculation consistently reduced the concentrations of catalpol in the shoots by respectively 48% and 53%, both when springtails were present and

absent (ANOVA: df=36, F=8.43, p<0.001; Tukey: for 2both p<0.05, Figure 11c). Inoculation with AM fungi had no effect on the catalpol concentrations in the root (ANOVA, df=36, F=0.93, p=0.44). Of the three secondary metabolites found in roots, the catalpol concentrations were the lowest: in 13 of the 40 root samples, catalpol levels were under the detection limit. While aucubin was detectable in all leaf and root samples, none of the treatments had a significant effect on the aucubin concentration in plant shoots (ANOVA: df=36, F=2.45, p=0.08) or roots (ANOVA: df=36, F=1.99, p=0.13, Figure 11b). The power analysis indicates that ~17 replicates would be required to detect a significant effect of the fungal treatment on the aucubin concentration of the roots.

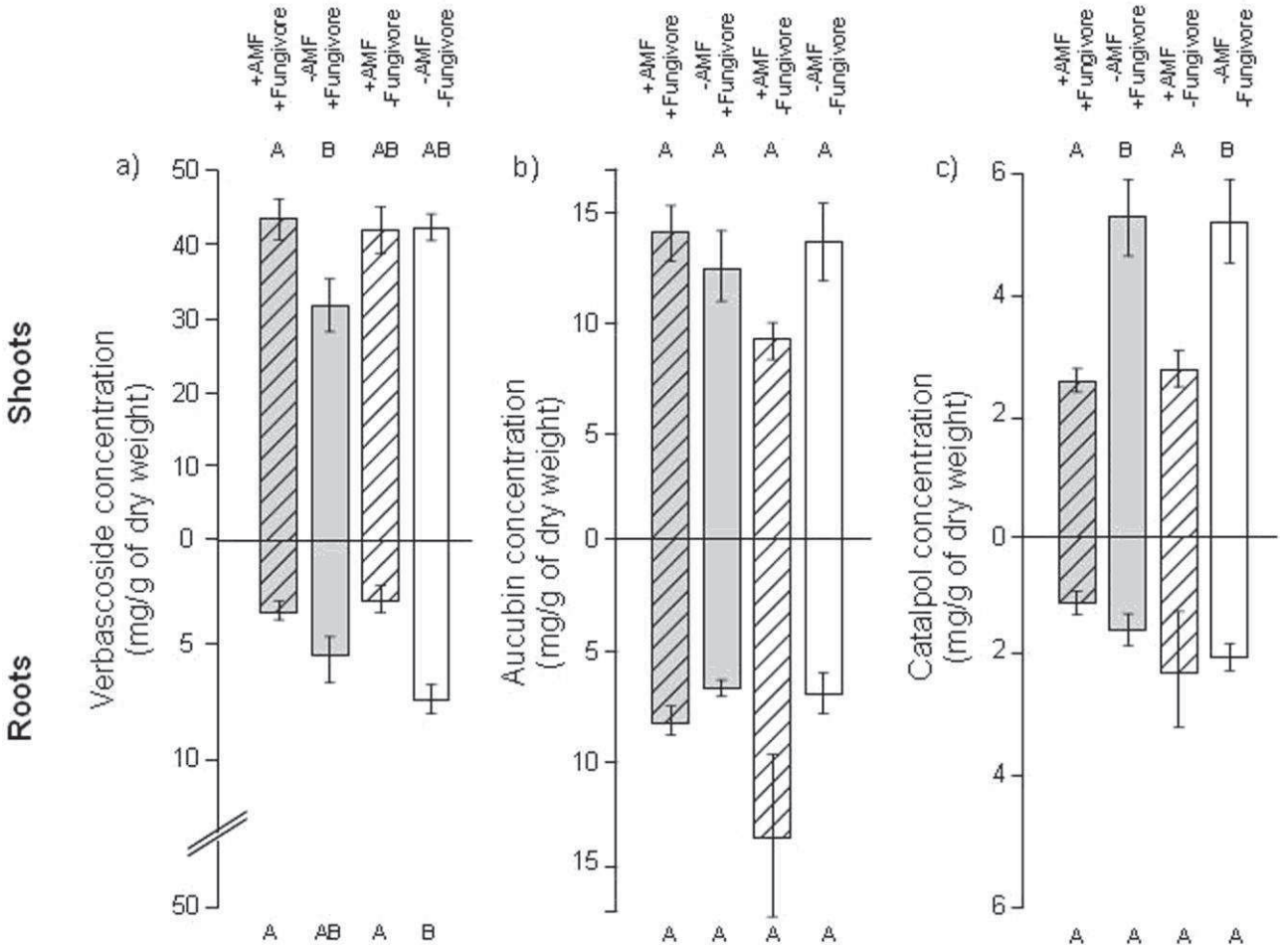


Figure 11:

Effects of AM fungal species *G. intraradices* (hatched bars) and the fungivore *F. candida* (grey bars) on (a) verbascoside (b), aucubin (c) and catalpol concentration in *P. lanceolata* shoots and roots (+SEM). Above the x-axis corresponds to aboveground concentrations, below the x-axis corresponds to belowground concentrations. Letters indicate significant differences between treatments means according to Tukey's HSD test (P ≤ 0.05). Bars represent the means of 10 replicates ± 1 standard error.

We measured the presence of secondary metabolites in AM fungal hyphae in treatments with and without springtails. While aucubin and verbascoside were undetectable in all hyphal samples, we consistently identified catalpol (mean concentration of 0.35±0.12 mg g-1 dry hyphae, Figure 12) in the fungal hyphae of each sample (n=6 because of pooling) from the treatment with springtails. In contrast, catalpol was undetectable in the fungal hyphae when no springtails were added. To confirm this finding, we also tested hyphae from the mixed fungal treatment (Appendix E), which due to inoculation failure of *G. custos*, only contained *G. intraradices*. Again, when springtails were present, AM fungal hyphae contained catalpol (mean concentration of 0.26±0.08 mg g-1 dry hyphae, n=7, Figure 12), while catalpol was undetectable when springtails were absent. As an initial test of whether the catalpol was produced by the fungus (e.g. in the absence of a photosynthetically active host), we tested the catalpol concentration of hyphae from in vitro root organ cultures. In all in vitro replicates, the catalpol level was under the detection limit (i.e. <250 ng ml-1).

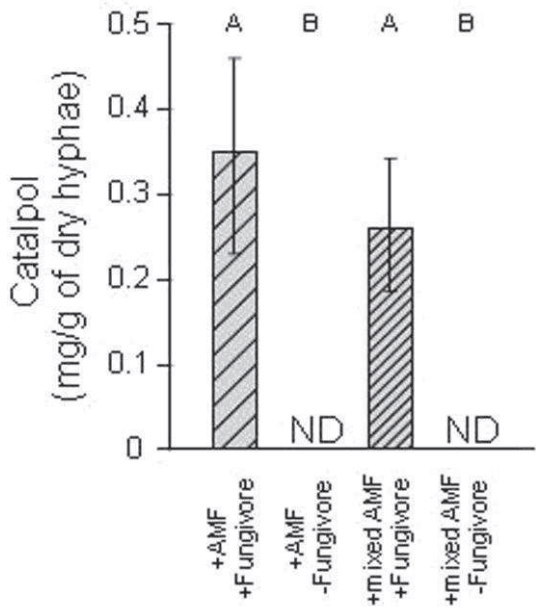


Figure 12:
Catalpol concentration in hyphae of *G. intraradices* (hatched bars) and in mixed treatment (tightly hatched bars) which contained *G. intraradices* (+ *G. custos* which failed to establish). Fungal treatments were either exposed to the fungivore *F. candida* (grey bars) or experienced no fungivores (white bars). Letters indicate significant differences between treatments means according to Tukey's HSD test ($P \leq 0.05$). Bars represent the means of 6 replicates (*G. intraradices* alone) or 7 replicates (mixed treatment) ± 1 standard error.

Lastly, we tested whether catalpol was a feeding deterrent for *F. candida* using food choice arenas. We found that at all levels tested (0.1%, 1% and 2% w/w), catalpol acted as an efficient repellent for the springtails. When catalpol was present, 79-91% (depending on concentration) of the springtails choose to feed from material on the unspiked, control side (Appendix F).

Discussion

Here we investigated the effects of fungivores on the concentration of secondary metabolites in shoots and roots of host plants, and hyphae of AM fungi. The most striking result of our study was that AM fungal hyphae contained catalpol (Figure 12). This iridoid glycoside was consistently identified in all hyphal samples exposed to springtails, suggesting that its presence is triggered by the presence of fungivores. secondary metabolites have been well-studied in plants but less is known about these compounds in fungi. Reported fungal secondary metabolites broadly fall into five diverse chemical categories: polyketides, polyketide-peptide hybrids, fatty acid derived compounds, amino acid-derived compounds and non-ribosomal peptides (Roze *et al.*, 2011). Previous work has identified secondary metabolites in Basidiomycota and Ascomycota phyla (Rohlfs & Churchill, 2011), and it is known that endophytic fungi can synthesize various secondary metabolites, like ergovaline, peramine, loline or indol derivatives (Fleetwood *et al.*, 2007; Yue *et al.*, 2000, Tanaka *et al.*, 2012). These compounds have been shown to negatively affect microarthropods (Rohlfs &Churchill, 2011), and exhibit antifungal and antimicrobial properties (Aly *et al.*, 2010). However, the secondary metabolite class of iridoid glycosides seem to be exclusive to the plant kingdom (Dinda *et al.*, 2007).

As this is the first evidence of secondary metabolites in AM fungal hyphae, it is not clear whether catalpol is synthesized by the plant or the fungus. As an initial test of this question, we collected hyphae from in vitro root organ cultures that lack a photosynthetic top. We did not find any evidence for secondary metabolites, suggesting that AM fungi do not synthesize catalpol de novo. However, these hyphae were not exposed to fungivores and thus iridoid secondary metabolites synthesis may not have been induced. While we utilized a different *G. intraradices* isolate than the one currently being sequenced, a preliminary search through the available genome data of *G. (Rhizophagus) intraradices* failed to provide any evidence for a functional biosynthetic pathway for iridoid glycosides in its genome. The fact that catalpol is one of the major secondary metabolites found in P. lancelota, and that there are no reports of catalpol being synthesized by other fungi in nature (Dinda *et al.*, 2007), is supportive of our hypothesis that catalpol is transferred by the host to the fungi to protect against springtails.

How is catalpol transferred to the hyphae? Recent work suggests that mycorrhizal networks can facilitate a transfer of allelopathic compounds - compounds produced by one

plant that limit the growth of surrounding plants (Barto *et al.*, 2011), but it is unknown if these compounds simply move along hyphal surfaces or whether they move inside hyphae. A transfer of biologically active secondary metabolites has been shown to exist in some root-hemiparasitic plants and their hosts, enabling these root parasites to reduce their susceptibility to herbivory by an uptake (via the haustorium) and sequestration of host-produced deterrents (Schädler *et al.*, 2005; Rasmussen *et al.*, 2006). The selective uptake and subsequent transport and storage of plant-derived secondary metabolites has also been found in several herbivorous insects (Leptidopterans, Coleopterans) to support anti-predator defense (Kuhn *et al.*, 2004). These observations all indicate the existence of specific mechanisms that enable the uptake and handling of “foreign” biologically active compounds without adverse effects on the organisms own physiological processes (e.g. involving “vesicle trafficking” in transfer/transport, see Field *et al.*, 2006). However, more work is needed to explore the movement and transfer (active or passive) of chemicals across hyphal networks.

A second result was that the hyphal biomass of the AM fungus was not reduced in the presence of springtails (Figure 10a). There was a trend towards reduced biomass in the presence of springtails, but this was never significant and the power analysis suggests that the lack of the significant difference is not the results of a small sample number. Biomass measurements were consistent with the qPCR analyses of roots and soil. Visually, we found the roots were well colonized ~50% root length colonized, and that this is the same or higher than root colonization rates reported for the field-grown *Plantago* plants (Šmilauerová & Šmilauer, 2002). No significant differences were found for the springtail treatments for any colonization data. Measurements of the mtLSU were used as a proxy for active fungal biomass (Alkan *et al.*, 2006), and again springtails did not lead to a significant reduction in fungal copy number (Figure 10b and c).

There are two potential explanations for why we do not see a significant reduction in hyphal biomass. First, it is possible that the survival of the springtails was low due to the lack of appropriate food sources. We added ~120 individuals kg-1 soil, which is within the range for natural densities (Petersen & Luxton, 1982; Moore *et al.*, 1984). However, previous greenhouse experiments have shown that springtail numbers under ~200 individuals kg-1 of soil result in no negative reduction of fungal growth, and can even stimulate fungal colonization (Giller, 1996; Bakonyi *et al.*, 2002). There could be a compensatory effect to grazing by the springtails, with fungi allocating more to hyphal

regeneration and increased mycelium turnover in presence of these fungivores. The second possibility is that we are seeing an interplay between two opposing factors: while AM fungal hyphae was the only food source for the springtails, it was also an undesirable food source (Klironomos & Ursic, 1998). While the effects of plant-derived secondary metabolites vary depending on fungivore (Larsen *et al.,* 2008), they are generally very strong feeding deterrents to herbivores (Biere *et al.*, 2004). For example, collembola prefer to graze fungi containing less secondary metabolites, even if they may contain less nutrients (Jørgensen *et al.*, 2005; Staaden *et al.*, 2010). Our food choice experiments demonstrate that catalpol is a strong repellent for *F. candida* when spiked in the springtail's regular laboratory food (baker's yeast) at concentrations < 0.1% w/w (Appendix F). So while we would expect a decrease in the AM fungal biomass as sole food source, the reduction may be less pronounced due to the repellant qualities of the hyphae themselves.

As expected, we found a positive effect of AM fungal colonization on plant biomass in the treatments with *G. intraradices* (Figure 9). However, we did not expect that the presence of springtails - in the absence of AM fungal colonization - would increase plant biomass (Figure 9). While one possible explanation is that dead springtails provided extra nutrients or other growth promoter, our calculations indicate that the nitrogen content in 200 springtails (~130 µg of N/pot) is insignificant compared to what was added as nutrient solution (~4.5 mg of N/pot). All growth data from hosts inoculated with *G. custos* were removed from the analysis because of the inoculation failure with this fungi. While we have had success with this AM fungal species in the past (e.g. Verbruggen *et al.*, 2012), the soil characteristics of our pot cultures (composition, pH, moisturing) were potentially not favorable for its growth.

Consistent with the results of other authors (Gange & West, 1994; De Deyn *et al.*, 2009), we found that inoculation with AM fungi resulted in changes in the secondary metabolite contents of plant shoots and roots (Figure 11). While secondary metabolite levels are known to vary depending on numerous factors like plant age, pathogen presence, AM fungal colonization, nutrient availability, and genetic factors (Marak *et al.*, 2002; Fuchs & Bowers, 2004; Barton, 2007), our secondary metabolite levels were in a similar range to those found by others in greenhouse experiments (Fajer *et al.,* 1992, shoots only, Fontana *et al.,* 2009, shoots only, De Deyn *et al.*, 2009, roots & shoots). In a manipulative experiment similar to ours, De Deyn *et al.* (2009) studied the effect of AM fungi on selected lines of *P. lanceolata*, containing high and low levels of iridoid glycosides. They found a catalpol range of 0.05%-0.8% and aucubin range of 0.05% - 1.0%, in the low

and high lines, respectively. These levels are in the range of our experiment, with catalpol levels found at 0.1% - 0.52% and aucubin at 0.6-1.4%. Also in agreement with a trend identified by De Deyn *et al.* 2009, we demonstrated that inoculation with AM fungi decreased catalpol levels in shoots (Figure 11c). We found that aucubin levels were unaffected by our experimental treatments (Figure 11b) and that colonization by AM fungi resulted in a decrease in 4the verbascoside levels in plant roots (Figure 11a). In the presence of springtails and absence of AM Fungi, the verbascocide concentration was lower than in any other treatment. A possible explanation would be that the reduced verbascoside production explains benefits to plant biomass. However, previous studies suggest that the costs of secondary metabolite products in *Plantago lanceolata* are minor (e.g. Darrow & Bowers, 1997), especially when nutrients are in short supply giving rise to a relative surplus on photosynthate available in the synthesis of the iridoids (Marak *et al.* 2003). Therefore explaining an 80 mg increase in plant biomass from a 2.5 mg saving in verbascoside content is probably unlikely. Previous work has shown increases in secondary metabolites in leaves after AM fungal colonization (Gange & West, 1994), or no effect at all (Wurst *et al.*, 2004; Fontana *et al.*, 2009), highlighting the variability of secondary metabolite synthesis. Levels of secondary metabolites may also be higher in field grown plants compared to greenhouse plants, potentially due to exposure to even more threats (e.g. Bower *et al.,* 1992). Changes in plant secondary metabolite levels can also be very local (Stout *et al.*, 1996; Darrow & Bowers, 1999), which explains how we can see variations in catalpol in the aboveground portions and variations of verbascoside in roots only.

Conclusion

Given the substantial investment of plants and fungi to form a mycorrhizal network, both partners have a shared interest in protecting it. Fungivores present a constant threat. What strategies do plant and/or fungus employ to safeguard hyphal network from grazing? Our results suggest that the plant may contribute to the chemical protection of the hyphal network. In the presence of fungivores, catalpol was found in the hyphae of AM fungi. When fungivores were absent, the catalpol concentrations in the hyphae were below the detection limit. This suggests that catalpol can be triggered by fungivore grazing pressure. As the synthesis of allelochemicals may involve costs, it is understandable why these

compounds are only found in the hyphae when there is a strong threat, such as fungivores.

Several aspects of the origin and transfer of protective compounds in hyphal networks warrant further study. For instance, we need more research to deduce whether AM fungi are capable of a deterrent metabolite synthesis of their own, and to test for the presence of a wider array of compounds such as mycorradicin and blumenin that may be transferred by mycorrhizal plant species as feeding deterrents (Maier *et al.*, 1995; Strack *et al.*, 2003 review). We also need a better understanding of the origin and/or transfer mechanisms of protective compounds, and whether compounds travel along the hyphae extracellularly (e.g. Barto *et al.*, 2011) or intracellularly, as we predict. Lastly, we utilized only one host, one AM fungal species and one fungivore. More work is needed to broaden these conclusions and determine whether this is a common strategy across mycorrhizal host plants.

Acknowledgements

We are grateful for the input of two anonymous reviewers and INRA-Nancy (F Martin's Lab of Excellence ARBRE, U.S. DOE Joint Genome Institute and *Glomus* Genome Consortium for access to the *Glomus* genome annotation. M. Duhamel and P. Vandenkoornhuyse were supported by ANR-ECS, J. Jansa was supported J.E.Purkyně, Czech Science Foundation (P504121665), and the long-term development program RVO 61388971, E.T. Kiers and H. Bücking by the National Science Foundation (award 1051397), E.T. Kiers by Dutch Science Foundation (NWO: meervoud 836.10.001 and vidi 864.10.005 grants) and R. Pel. By NWO (Open Programme 822.01.003).

Supplemental Material:

Methodological Details Of Plant Propagation/set-up, Nutrient Regime, Hyphal Chemical Analysis (Appendix A)

QPCR Methodology And Probe Design (Appendix B)

Summary Table Of Results And Statistics (Appendix C)

Comparison Of ANOVA And GLM Analyses For Biomass Measurements And Secondary Metabolites In Roots And Shoots (Appendix D)

Dry Weights Of Fungal Hyphae From ‘mixed’ Fungal Treatment (Appendix E)

Choice Experiment Data Testing Catalpol Palatability On *F. Candida*. (Appendix F)

Appendix A: Methodological details of plant propagation/set-up, nutrient regime, hyphal harvest and chemical analysis

Plant propagation

Before seedlings were planted in bags, the mesh bags were washed with demineralized water, and sterilised with 70% ethanol. Each mesh bag contained 300 g of a sterilized 9:1 mixture of quartz sand mixed with organic dune sand (see Verbruggen *et al.,* 2012 for soil description), resulting in 0.8% of organic matter per bag (15% humidity). All pots were covered with black plastic foil to avoid evaporation.

Nutrient solution

The nutrient solution was injected below the quartz sand surface (over a depth of 1-8 cm) at 6 equidistant positions around the meshbag to achieve an even extraradical mycelium (ERM) development, and to minimize the growth of algae on the sand surface of the pots.

Hyphal Harvest

To measure the hyphal mass, blocks of sand were removed from the pot. The sand portion on the sieve was partly submerged in demineralised water and gently shaken to allow the sand to pass, leaving the intact ERM with quartz grains still attaching to the hyphal surface. By applying a gentle flow of demineralised water a portion of these attached grains could be removed.

To extract the hyphae from *in-vitro* medium, the fungal compartment of the medium was cut in 5-6 pieces and transferred in a 50 ml tube together with 25 ml 10mM sodium citrate buffer (pH 6, 37 °C). The solution was vortexed for 30 minutes at 100 rpm and sieved with nylon mesh. The washing steps were repeated until all medium was washed away. Roots were removed by hand using a stereomicroscope and tweezers. Hyphae were put in an eppendorf tube, freezed it in liquid nitrogen and stored in a -80 °C freezer until analysis**.**

Chemical analysis

Quantitative HPLC analysis was conducted using an Ultimate 3000 system (Dionex, USA) consisting of a dual pump module, autosampler, column compartment and photodiode array detector. A LiChroCART 125-4 LiChrospher 100RP-18 (5 µm) (Merck,

Darmstadt, Germany) was used as analytical column. The mobile phase consisted of (A) phosphate buffer (2.5 mM KH_2PO_4; pH= 4.0 with phosphoric acid) and (B) acetonitrile. The gradient elution had the following profile: 0-0.2 min, 0% B; 0.2-10.5 min, 0-6% B; 10.5-24.5 min, 6-50% B; 24.5-26.5 min, 50-0% B; 26.5-29 min, 0% B. The column flow rate was 1 ml min^{-1} at 20 ºC, and the injection volume was 10 µl. The absorbance of catalpol and aucubin were measured at 204 nm, and verbascoside was measured at 215 nm. SM peaks in the chromatograms were identified by comparison of retention time of authentic iridoid and verbascoside standards (Carl Roth GmbH, Germany): catalpol (purity 99%) at 4.8 min, aucubin (purity 98,5%) at 7.6 min and verbascoside (purity 98%) at 17.9 min. The peak area was integrated using Chromeleon Software Release 6.60 (Dionex Corp.) with external standards. Standard calibration curves were plotted using various concentrations of catalpol, aucubin and verbascoside (range: 10 – 250 µg ml^{-1} for each compound). The detection limit of catalpol and aucubin was 250 ng ml^{-1} and 80 ng ml^{-1} for verbascoside.

Statistical analysis

The functions used in R to perform the statistical analyses are the following:

- normality and homogeneity of variances: ks.test(); levene.test()
- analyses of plant data, hyphal biomass and secondary metabolite concentrations: glm(); anova(lm()); TukeyHSD()
- molecular analyses and data on secondary metabolites in hyphae: t.test(); welch.test()
- power analyses: power.anova.test(); power.t.test()

For GLM analysis, the best possible GLM was selected after calculation of Akaike Information Criterion. In this study, the most appropriate family wise errors were Inverse Gaussian or Gaussian depending on the data. Modalities were included in the GLM one at a time. From the GLM, data were analysed by a one-sample t-test.

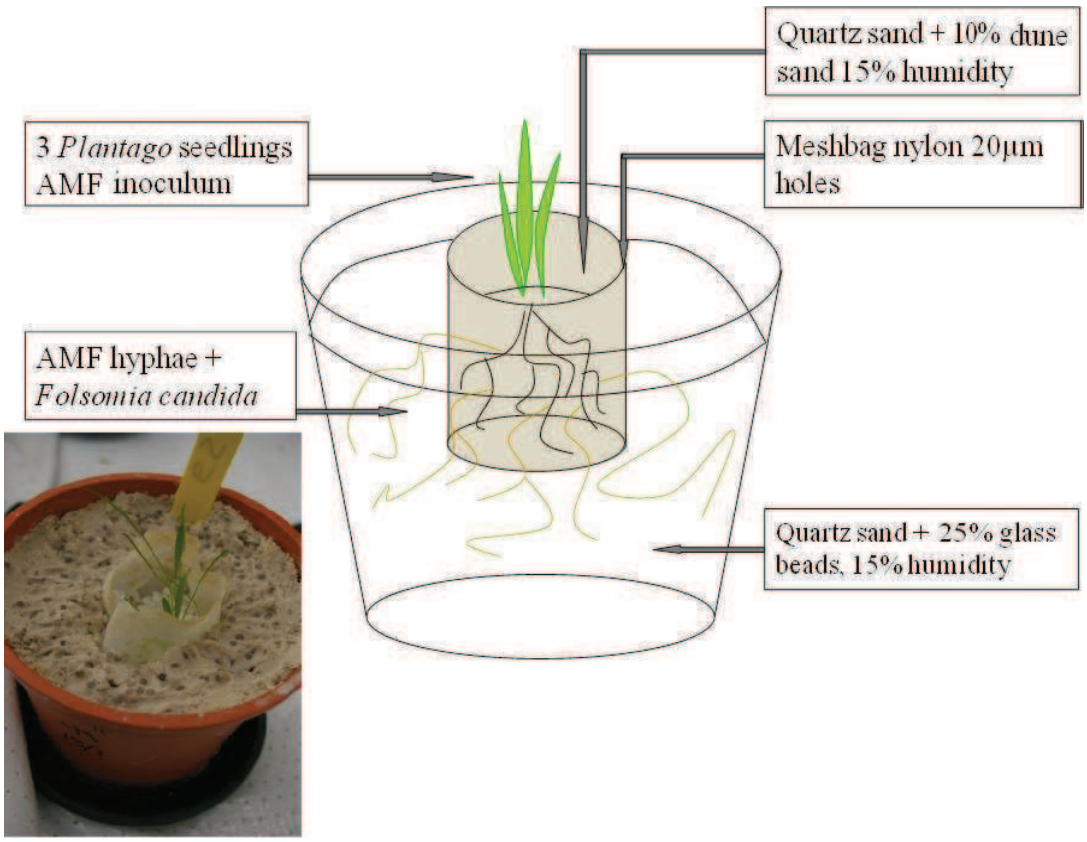


Figure A1:
Experimental set-up.

Appendix B: Methodological qPCR analyses details

qPCR assays were run in 9 µl reactions, using the LightCycler 2.0 instrument (Roche), 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 and0.11 µM, respectively (for sequences see Table B1). Each reaction included 2.25 µl of the DNA sample.

Table B1:
qPCR markers for specific quantification of *Glomus intraradices* and *G. custos* by measuring gene copies of the mitochondrial large ribosomal subunit. *FAM* – fluorescein, *BHQ1* – fluorescence quencher.

Fungus	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***-AAACTGCCACTCCCTCCATATCCAA-***BHQ1***	65	95 / 10	60 / 10	72 / 1
Glomus custos	TCTAACCCCAGAAATGTATAG, AAGGACTGCCTTGTGTTC, ***FAM***-ATACAATAATGGGCAATCAGACATATCGT-***BHQ1***	65	95 / 10	62 / 15	72 / 1

Appendix C: Summary table of results and statistics

Appendix C Legend	*G. intraradices*	Fungivores
C	-	-
S	-	+
GI	+	-
GIS	+	+
M	+	-
MS	+	+

		N	MEAN	SEM	p value
Shoots biomass (g)	C	10	0.177	0.004	ANOVA and GLM see Appendix D
	S	10	0.250	0.011	
	GI	10	0.283	0.008	
	GIS	10	0.309	0.018	
Roots biomass (g)	C	10	0.213	0.004	ANOVA and GLM see Appendix D
	S	10	0.219	0.016	
	GI	10	0.231	0.047	
	GIS	10	0.272	0.013	
Hyphae biomass (g)	GI	10	0.022	0.004	Welch t = -0.92, df = 17.72, p-value = 0.36 Student t = 0.92, df = 18, p-value = 0.36
	GIS	10	0.016	0.005	
	M	10	0.020	0.005	Welch t = -0.09, df = 14.42, p-value = 0.92
	MS	10	0.019	0.003	
qPCR Roots (copy number/g)	GI	7	1.229E+09	6.320E+07	Welch t = 0.36, df = 6.84, p-value = 0.72 Student t = 0.36, df = 12, p-value = 0.72
	GIS	7	1.139E+09	2.377E+08	
qPCR Soil (copy number/g)	GI	7	2.806E+05	2.460E+04	Student t = 0.3, df = 12, p-value=0.76
	GIS	7	2.529E+05	8.803E+04	
Catalpol Shoots (mg/g)	C	10	5.169	0.694	ANOVA and GLM see Appendix D
	S	10	5.261	0.641	
	GI	10	2.764	0.309	
	GIS	10	2.572	0.205	
Catalpol Roots (mg/g)	C	10	1.640	0.224	ANOVA and GLM see Appendix D
	S	10	1.512	0.278	
	GI	10	2.207	0.958	
	GIS	10	1.084	0.202	

Catalpol Hyphae (mg/g)	GIS	6	0.349	0.116	
	S	7	0.259	0.079	
Aucubin Shoots (mg/g)	C	10	13.668	1.727	ANOVA and GLM see Appendix D
	S	10	12.406	1.646	
	GI	10	9.146	0.870	
	GIS	10	14.081	1.315	
Aucubin Roots (mg/g)	C	10	7.074	0.971	ANOVA and GLM see Appendix D
	S	10	6.881	0.408	
	GI	10	13.637	3.816	
	GIS	10	8.436	0.709	
Verbascoside Shoots (mg/g)	C	10	42.412	1.916	ANOVA and GLM see Appendix D
	S	10	31.831	3.670	
	GI	10	42.198	3.334	
	GIS	10	43.715	2.902	
Verbascoside Roots (mg/g)	C	10	7.245	0.697	ANOVA and GLM see Appendix D
	S	10	5.281	1.069	
	GI	10	2.718	0.623	
	GIS	10	3.277	0.463	

Appendix D: Comparison of ANOVA and GLM analyses for biomass measurements and secondary metabolites in roots and shoots.

	ANOVA (treatment effect)			ANOVA (Fungi and Fungivore effect, interactions)				GLM				
	df	F	p-value		df	F	p-value	Family wise error		df	F	p-value
Shoots biomass	3	24.027	1.029e-08	Fungi	1	50.1758	2.552e-08	gaussian	Fungi	1	67.113	9.592e-10
				Fungivore	1	17.7872	0.0001		Fungivore	1	21.735	4.199e-05
				Interaction	1	4.1183	0.0498		Interaction	1	24.683	1.656e-05
Roots biomass	3	1.2736	0.2985	Fungi	1	0.0205	0.8871	inverse. gaussian	Fungi	1	1.7014	0.2006
				Fungivore	1	1.9028	0.1765		Fungivore	1	0.7922	0.3795
				Interaction	1	1.8976	0.1771		Interaction	1	0.4443	0.5094
Catalpol in Shoots	3	8.4336	0.0002	Fungi	1	25.2126	1.408e-05	inverse. gaussian	Fungi	1	35.588	7.725e-07
				Fungivore	1	0.0100	0.9211		Fungivore	1	0.0096	0.9226
				Interaction	1	0.0784	0.7811		Interaction	1	0.2935	0.5913
Catalpol in Roots	3	0.9297	0.4364	Fungi	1	1.5178	0.2259	inverse. gaussian	Fungi	1	0.0308	0.8618
				Fungivore	1	1.0492	0.3125		Fungivore	1	2.5673	0.1178
				Interaction	1	0.2219	0.6404		Interaction	1	1.7192	0.1981
Aucubin in Shoots	3	2.4451	0.0797	Fungi	1	0.9909	0.3261	inverse. gaussian	Fungi	1	0.9909	0.3261
				Fungivore	1	1.6492	0.2072		Fungivore	1	1.6492	0.2072
				Interaction	1	4.6952	0.0369		Interaction	1	4.6952	0.0369
Aucubin in Roots	3	1.9874	0.1333	Fungi	1	4.7049	0.0367	gaussian	Fungi	1	9.7448	0.0035
				Fungivore	1	0.5317	0.4706		Fungivore	1	3.6585	0.0637
				Interaction	1	0.7255	0.3999		Interaction	1	0.7335	0.3974
Verbascoside in Shoots	3	4.4054	0.0097	Fungi	1	4.1761	0.0483	gaussian	Fungi	1	3.7130	0.0619
				Fungivore	1	3.1788	0.0830		Fungivore	1	2.2398	0.1432
				Interaction	1	5.8615	0.0206		Interaction	1	3.9893	0.0533
Verbascoside in Roots	3	7.6236	0.0004	Fungi	1	19.1284	9.997e-05	gaussian	Fungi	1	19.1284	9.997e-05
				Fungivore	1	0.8849	0.3531		Fungivore	1	0.8849	0.3531
				Interaction	1	2.8575	0.0996		Interaction	1	2.8575	0.0996

Appendix E: Dry weights of fungal hyphae from 'mixed' fungal treatment

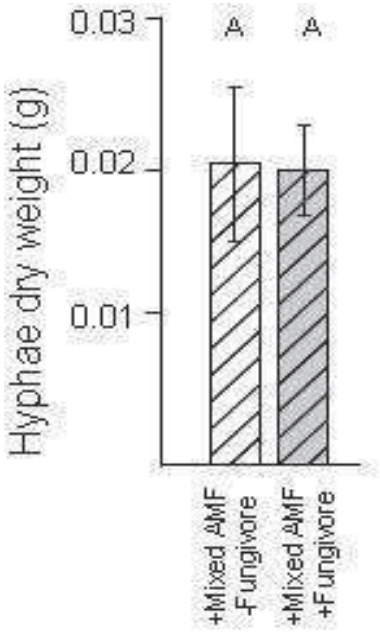


Figure E1:
Effect of F.candida presence (grey) and absence (white) on the mean dry weights of fungal hyphae collected outside the meshbags for the treatments with AMF mixture (Bars represent the means of 10 replicates ± 1 standard error). Letters indicate significant differences between treatments means according to Tukey's HSD test ($P \leq 0.05$).

Chapter III

PHYMYCO-DB: A Curated Database for Analyses of Fungal Diversity and Evolution

Stéphane Mahé1, Marie Duhamel13, Thomas Le Calvez, Laetitia Guillot, Ludmila Sarbu, Anthony Bretaudeau, Olivier Collin, Alexis Dufresne1, E. Toby Kiers3, Philippe Vandenkoornhuyse1

1Universite ´ de Rennes I, CNRS, UMR 6553 ECOBIO, Campus de Beaulieu, Rennes, France, 2Universite ´ Européenne de Bretagne, Rennes, France, 3Department of Ecological Science, Vrije Universiteit, Amsterdam, The Netherlands, 4Universite ´ de Rennes I, CNRS, UMR 6074 IRISA, Campus de Beaulieu, Rennes, France, 5Centre Scientifique et Technique du Batiment, AQUASIM, Nantes, France

PLoS ONE 7(9): e43117 2012

Abstract

Background

In environmental sequencing studies, fungi can be identified based on nucleic acid sequences, using either highly variable sequences as species barcodes or conserved sequences containing a high-quality phylogenetic signal. For the latter, identification relies on phylogenetic analyses and the adoption of the phylogenetic species concept.

Such analysis requires that the reference sequences are well identified and deposited in public-access databases. However, many entries in the public sequence databases are problematic in terms of quality and reliability and these data require screening to ensure correct phylogenetic interpretation.

Methods and Principal Findings

To facilitate phylogenetic inferences and phylogenetic assignment, we introduce a fungal sequence database. The database PHYMYCO-DB comprises fungal sequences from GenBank that have been filtered to satisfy stringent sequence quality criteria. For the

first release, two widely used molecular taxonomic markers were chosen: the nuclear SSU rRNA and EF1-α gene sequences. Following the automatic extraction and filtration, a manual curation is performed to remove problematic sequences while preserving relevant sequences useful for phylogenetic studies. As a result of curation, ~20% of the automatically filtered sequences have been removed from the database. To demonstrate how PHYMYCO-DB can be employed, we test a set of environmental Chytridiomycota sequences obtained from deep sea samples.

Conclusion

PHYMYCO-DB offers the tools necessary to: (i) extract high quality fungal sequences for each of the 5 fungal phyla, at all taxonomic levels, (ii) extract already performed alignments, to act as 'reference alignments', (iii) launch alignments of personal sequences along with stored data. A total of 9120 SSU rRNA and 672 EF1-α high-quality fungal sequences are now available.

The PHYMYCO-DB is accessible through the URL http://phymycodb.genouest.org/.

Introduction

In recent years there has been an exponential increase in the number of gene sequences available in public-access databases. This is the result of new developments in molecular techniques and new generation sequencers that allow the collection of data at great speed. The use of molecular taxonomic markers associated with phylogenetic analyses has revealed considerable genetic diversity in fungi, especially those that are cryptic, unculturable or not easily distinguishable by morphological characters (e.g. Vandenkoornhuyse *et al.,* 2002a). As the species concept is employed for diversity measurements, systematics and evolutionary analyses (Purvis & Hector, 2000), an efficient means of identifying boundaries, and thus number of species, is required. Molecular methods and the implicit adoption of the phylogenetic species concept (Taylor *et al.,* 2000) offer a standardized approach to delimit groups of organisms (e.g. Vandenkoornhuyse *et al.,* 2002b*;* Jones *et al.,* 2011; Powell *et al.*, 2011). Thanks to progress in sequencing technologies and bioinformatic methods, the detection of orthologous sequences using databases is relatively efficient. This approach can also be successfully applied to organisms that are not available in culture, increasing our ability to identify new diversity in various habitats (Hawksworth & Rossman, 1997; Blackwell, 2011). Of course, this approach requires choosing a relevant molecular marker which: (i) targets a nucleic acid sequence with a limited proportion of homoplasy (i.e. correspondence between parts arising from evolutionary convergence), (ii) contains high phylogenetic information which is not sensitive to paralogy (i.e. single copy genes or highly conserved genes). This allows for accurate characterization of evolutionary affinities.

In this context, the nuclear gene coding for the small subunit of the ribosomal RNA (SSU rRNA) is often seen as the ‘ultimate’ molecular marker (Woese, 2000) (for review Pace, 2009). The SSU rRNA gene is present in all living organisms. Its sequence is highly conserved between taxa, reflecting strong functional constraints on the translational machinery. Indeed, most mutations in the SSU rRNA gene sequence reduce the stability of the secondary structure of the SSU rRNA molecule and thus the efficiency of protein synthesis. Furthermore, this gene, like other informational genes, appears to be less subject to horizontal gene transfers and is believed to provide better inferences of ‘true’ phylogenies (Choi & Kim, 2007). Although the SSU rRNA gene can have a multicopy status within a single fungal genome, sequence variations have been shown to be

extremely low or null. For example, from available complete annotated genomes (http://www.genomesonline.org/cgi-bin/GOLD/index.cgi), *Saccharomyces cerevisiae* has two SSU rRNA copies both on its chromosome XII. *Encephalitozoon cuniculi*, a Microsporidia, has two SSU rRNA genes copies one on its chromosome I, the other on chromosome IV. In these two cases, the copies display 100% identity. This is not surprising since the SSU rRNA gene is highly conserved. Thus this gene is less sensitive to paralogy compared to LSU rRNA gene and ITS where variations among copies have been clearly shown (e.g. Boon *et al.*, 2010; Sanders *et al.,* 1995; Lim *et al.*, 2008).

A second advantage of using the SSU rRNA gene sequence is its huge representation in international public databases - GenBank (Benson *et al.*, 2011*)*, EMBL/ENA (Kulikova *et al.*, 2007), DDBJ (Kaminuma *et al.*, 2011) – which facilitates comparisons between a wide variety of organisms (for review Avise, 2004). One disadvantage is that because the SSU rRNA gene is highly conserved, the resolution of the phylogenetic analyses is poor for youngest fungal groups within Ascomycota. Other genes, such as those encoding for the elongation factor EF1-α (*tef1*), for β-tubulin (*tub1, tub2*), actin (*act1*), or for RNA polymerase II subunits (*rpb1* and *rpb2*), can be used as alternative markers. Among these ones, EF1-α sequence data are the most abundant but only represent a small fraction of the amount of SSU rRNA yet available (i.e. less than 7% of the total number of sequences contained in PHYMYCO-DB). Generally present as a single copy gene, the EF1-α gene is involved in protein synthesis and displays a higher mutation rate than SSU rRNA gene. Because of these attributes, EF1-α protein sequences have been used to resolve phylogenetic affinities between eukaryotic organisms (Baldauf, 1999; Baldauf *et al.*, 2000; Helgason *et al.*, 2003), and particularly the sister clade relationship of animals and fungi (Baldauf & Palmer, 1993). The gene sequences also have the potential to help resolve phylogenetic relationships between closely related fungi (Helgason *et al.*, 2003 ; Moon *et al.*, 2002 ; Tanabe *et al.,* 2004), but they contain a higher proportion of homoplasious positions compared to SSU rRNA gene sequences. Studies of both SSU rRNA genes and EF1-α genes could greatly improve the resolution of fungal phylogenetic affinities. An online database incorporating data from both these sequences is a key step to achieving improved phylogenetic resolution for fungi.

Pollution of public sequence database and the aim of PHYMYCO-DB

One major obstacle for international public databases is constant pollution by non-negligible proportions of compromised sequences (GenBank/EMBL/DDBJ). This problem, discussed in several articles and journal forums (e.g. Bidartondo, 2008; Bridge *et al.*, 2003 ; Vilgalys, 2003 ; Bridge *et al.*, 2004 ; Hawksworth, 2004 ; Hawksworth, 2009 ; Holst-Jensen *et al.*, 2004 ; Nilsson *et al.*, 2006), is becoming more and more obvious, but solutions remain elusive. Problematic data can arise from many different origins, including: (i) erroneous specimen identification (Vilgalys, 2003), (ii) the use of separate names for different sexual stages (Hawksworth, 2009), (iii) differences in taxonomy among specialists (Vilgalys, 2003) and/or advances in knowledge since the time the sequence was deposited leading to wrong designations (Hawksworth, 2004), (iv) the lack of precision in the description of the deposited sequences making their interpretation difficult (Kõljalg *et al.*, 2005), (v) sequences resulting from artefactual origin (i.e. chimeric sequences), and (vi) sequences of poor quality with undefined positions. Even more problematic is the erroneous annotated sequences that propagate within open access databases because of phylogenetic misinterpretation. Additionally, more and more sequence assignments are based solely on identity searches using heuristic local alignment (i.e. BLASTn searches). All these mistakes have the potential to jeopardize interpretations. Therefore, assessing the reliability of sequences is an increasingly important prerequisite to analyses.

Many of these errors can be limited via expert curation. Expert curation is critical for the continued advancement of the field because it allows for the production of sequence databases, containing accurate and reliable sequences. To date, most curated databases specialize in particular taxonomic groups (e.g. Öpik *et al.*, 2010), collect data associated to each nucleic acid sequence, and work with specimens validated by experts and deposited in public reference collections (e.g. Kõljalg *et al.*, 2005). Several important tools, such as the Ribosomal Database Project (Cole *et al.*, 2009), SILVA (Pruesse *et al.*, 2007), Greengenes database (DeSantis *et al.*, 2006) exist online for the analysis of SSU rRNA gene sequences. Apart from SILVA, these databases use automated filters to remove part of the polluting sequences. However, manual curation is an essential component of these projects and should aim to be even more stringent.

Based on lessons learned from other curated databases, our aims at PHYMCO-DB are to: (i) develop an easy-to-use fungal-dedicated database with stored sequences of

high quality, (ii) use selected molecular markers that are widely acknowledged, namely SSU rRNA and EF1-α, (iii) produce a tool, based on anchor sequences covering the fungal tree, that can be automatically updated, along with an expert curation of the new sequences, (iv) produce high quality multiple alignments for use in testing environmental sequences or evolutionary hypotheses.

Database Structure : Design and Implementation

The sequences constituting PHYMYCO-DB version 1 (Figure 13) were retrieved in October 2011 from the release 185 of GenBank (NCBI). The nuclear SSU rRNA and EF1-α genes sequences are extracted from the GenBank database, using the following queries: “[organism] and (ssu|SSUrRNA|SSU rRNA|18SrRNA|18S|) not (16S|mitoch*|28S|5.8S|ITS|Internal Transcribed Spacer|internal transcribed spacer|)” and “[Organism] and (EF1 alpha|EF-1 alpha|EF1-alpha|EF-1alpha|EF-1-alpha|EF1 alpha|EF1a|)”.After this extraction step, automatic quality filter parameters are applied. For SSU rRNA, nucleic acid sequences that are shorter than 1000 nucleotides and longer than 2500 nucleotides are rejected. Likewise for EF1-α genes, sequences shorter than 700 nucleotides and longer than 2500 nucleotides are discarded. Also sequences containing more than 10 consecutive undetermined nucleotides are excluded. According to the automatic quality criteria, all accepted sequences are then stored in a MySQL 5 relational database. The MySQL table structure is presented as a figure available in supplementary online information (Figure S1). PHYMYCO-DB is automatically updated 4 times a year and is managed by administrators using the web interfaces developed with PHP version 4 programming language.

Following automatic filtering, datasets are then cross-checked by expert curators (hereafter ‘expert curation’). Multiple alignments are performed using Clustal X 2.1 (Larkin *et al.*, 2007) on small sequence groups (<400 sequences), which are closely related to obtain a high-quality alignment and to make the expert curation as accurate as possible. Sequences are deleted from the alignment and from the database in a manual cleaning process if they contain: errors of sequencing (i.e. containing several substitutions that are not found anywhere else, Figure 14), errors in the annotation (i.e. a sequence with a naming inside a different group, Figure 14), homopolymers insertions (Figure 14), many

undetermined nucleotides (Figure 14), erroneous alignment or reverse complementary sequences (Figure 14). This expert curation is time consuming but essential to obtain reliable sequences and high-quality alignments. By adopting strict rules of expert curation, subjectivity and mistakes become minimal. Following expert curation, species redundancy (i.e. identical sequences) are retained in the database to keep sequences arising from different origin and ecological settings. The detection of dubious sequences from the alignments does not result in correction of the sequence in international databases. They are, however, all removed from PHYMYCO-DB. When corrections are made for a given sequence, a new registration number is provided by GenBank for example. In this case, the corrected sequence will be automatically extracted (i.e. 4 updates per year) and will be examined by one of the expert curators.

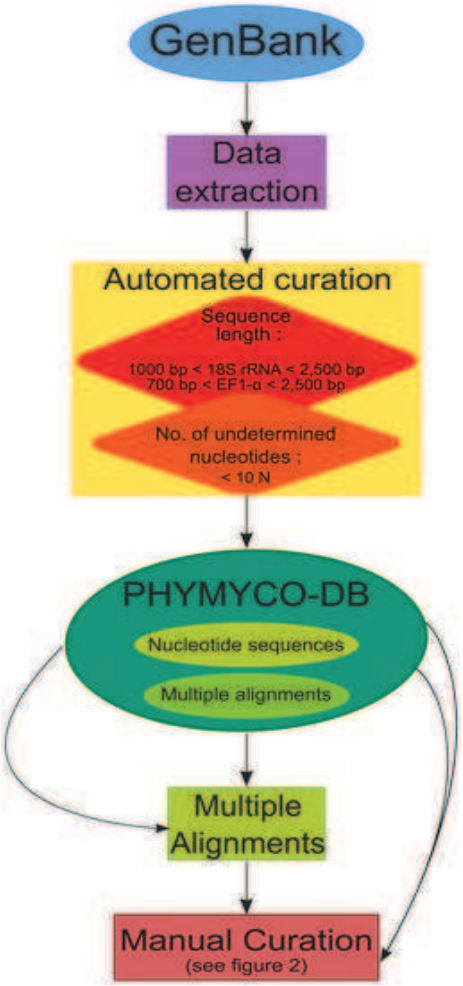


Figure 13:

Flowchart of the data in the PHYMYCO-DB. The arrows indicate the flow of gene sequences extracted from the GenBank database, through the automated and manual curation steps. All the sequences made available to users has passed the 2 curation processes. After each upgrade of the database (i.e. 4 times per year), expert manual curation is performed.

During our development process, it became clear that our automatic filters were not stringent enough to retrieve only trustworthy sequences. For example, SSU rRNA can present intron-like regions which could also be chimeric insertions. Introns are abundant in particular lineages of fungi, especially within lichen-forming fungi (Ascomycota). These fungi can display up to eight introns in the SSU rRNA gene, as for example found in the taxon Physconia (Bhattacharya *et al.*, 2002). At the expert curation stage, we noticed that the position of introns was not consistently given in the deposited sequence description, and they were detectable after the alignment only. When a sequence containing non-positioned introns was the only sequence of a particular genus, this sequence was kept, Otherwise the sequence was discarded from PHYMYCO-DB. Employing our curation principles, we discarded 2090 additional unreliable sequences, i.e. 18% of the sequences extracted from GenBank.

Following the curation steps, 8757 SSU rRNA gene sequences have been stored in PHYMYCO-DB (5088 Ascomycota, 2088 Basidiomycota, 366 Chytridiomycota, 1046 Glomeromycota, and 532 Zygomycota). PHYMYCO-DB also contains 648 EF1-α gene sequences (294 Ascomycota, 189 Basidiomycota, 10 Chytridiomycota, 25 Glomeromycota, and 154 Zygomycota). Our database contains less fungal sequences than SYLVA because of the level of curation stringency. All fungal genera has at a minimum one representative sequence within PHYMYCO-DB. Because of the heterogeneity among the number of sequences per taxonomic rank, and because we wanted a limited number of sequences for each alignment, the taxonomic level within these alignments is variable (family to phylum level). We therefore produced a total of about 50 ‘reference’ alignment files. These online alignments contain mainly full-length sequences, even if rare, very long sequences were cut at the same length as the others. This was done to keep maximum information available. This is especially useful for designing primers, and to give a greater freedom for manipulation by online users.

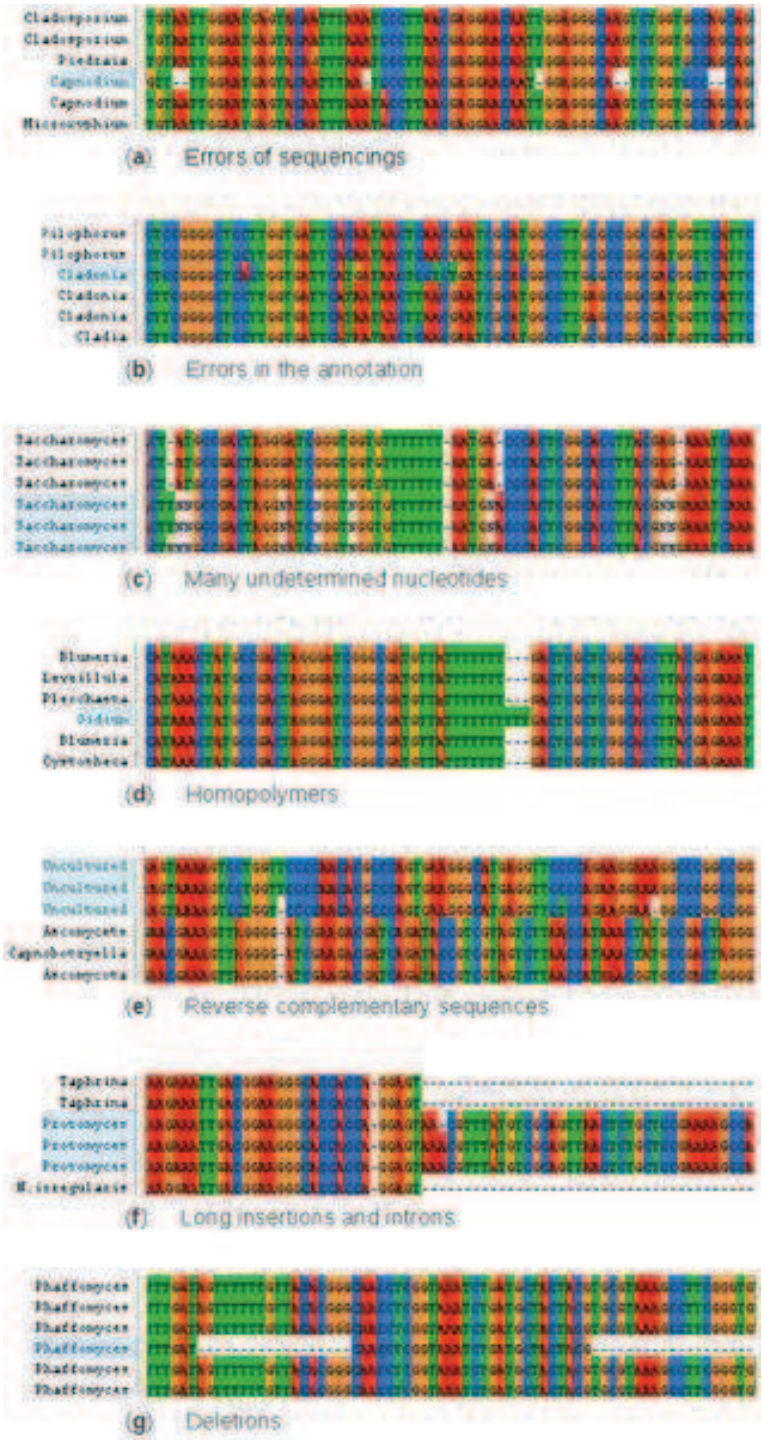


Figure 14 :

Visualisation of sequences deleted by the manual curation after alignment (ClustalX 2.1). The sequences highlighted in blue illustrate examples of sequences removed from PHYMYCO-DB. The compromised nature can stem from erroneous sequencing (e.g. repeated gaps), wrong annotation (e.g. sequence corresponding to another clade), high numbers of undetermined nucleotides, homopolymers insertions, erroneous alignment or reverse complementary sequences and presence of long insertions and introns or presence of deletions.

Tools within PHYMYCO-DB

We designed PHYMYCO-DB with specific tools to facilitate online use. Firstly, users can easily select sequences by browsing our interface through hierarchical taxonomic lineages presented in an arborescent structure (GenBank taxonomy), and then download them in a FASTA format file. The number of sequences stored in the database for each taxonomic level is given in brackets. Secondly, users can download an alignment file using a filter to find an alignment with the gene and the taxonomic rank requested. Special attention must be paid to the fact that some sequence characteristics in PHYMYCO-DB format are inherited from the extraction of GenBank sequences. For example, in some cases (e.g. Agaromycotina, a subphylum of Ascomycota), information on sequences taxonomy was associated to a 'no rank' tag in GenBank. To avoid the problem that these sequences are mistakenly placed in another taxonomic group, they were qualified as 'undefined' at the subphylum rank in PHYMYCO-DB. For the next lower taxonomic rank, no known tag problem exists. Environmental sequences have, by definition, no clear taxonomic ranking. Therefore, they were also qualified as 'undefined', but only until the lowest taxonomic rank. These are important features to take into account when using the PHYMYCO-DB.

Thirdly, users can launch a ClustalW 2.0 alignment on our back-end computer clusters by uploading their own personal sequences in a FASTA or ALN format file. A future PHYMYCO-DB release will offer the possibility to select the multiple alignment tool (i.e. ClustalW, MUSCLE, and MAFFT). Currently, users can choose to append an outgroup or sequences from a particular PHYMYCO-DB taxonomic group. We anticipate that this tool will be very efficient when combined with phylogenetic analyses for investigating the sequence diversity of fungal amplicons from an environmental sample and even to identify new fungal lineages.

PHYMYCO-DB as a Tool for Phylogenetic Identifications and Inferences

Based on a well-developed theoretical corpus, phylogenies can be computed using several different approaches (e.g. Felsenstein, 2004). From a mathematical point of view, the maximum likelihood phylogenetic reconstruction provides the best possible tree for a given explicit sequence evolution model. The model that best fits the aligned sequence

data can be selected, after using the popular Modeltest (Posada & Crandall, 1998). Achieving a good alignment is therefore of tremendous importance for good interpretation. Alignments should be refined using an 'influence function' that allows the removal of outlier columns from the matrix (i.e. nucleotide position where the phylogenetic signal differs from the general phylogenetic information recorded in the dataset, Bar-Hen*et al.*, 2008). This approach allows for a 'blind detection' of outliers using measures of each site in a context of a ML phylogenetic reconstruction. It must be emphasized that the sequence-based identification using SSU rRNA gene could be at the species level or at higher taxonomic levels depending on the fungal affiliation.

Following the above strategy, we provide an analysis of chytrid diversity as a proof of concept. Sequencing of the SSU rRNA gene was achieved by targeting chytrids from deep marine hydrothermal samples (ciPCR). First, the alignment of SSU rRNA gene sequences of the Chytridiomycota from PHYMYCO-DB were used to design specific primers manually. Two sets of designed primers covered the V3 and V4 variable regions and were suitable for pyrosequencing: C130 (5′TACCTTACTACTTGGATAACCG3′) with SR8R (5′TCAAAGTAAAAGTCCTGGATC3′) modified from Vilgalys lab webpage (http://www.biology.duke.edu/fungi/mycolab/primers.htm), and MH2 (5′TTCGATGGTAGGATAGAGG3′) (Vandenkoornhuyse & Leyval, 1998) with SR8R. Another set of primers, expected to be universal for fungi and to produce longer amplicons, were also tested: MH2 with NS7R (5′ATCACAGACCTGTTATTGCC3′) modified from (White *et al.*, 1990). Primers specificity was checked with a sample from a hydrothermal site from which several sequences of chytrids were retrieved (Le Calvez *et al.*, 2009). The resulting sequences (GenBank accession numbers JN986721 to JN986723) were analyzed using the corresponding 'reference' alignment in PHYMYCO-DB and the sequences having the highest similarity score in BLASTn. The computed phylogeny highlights the presence of a new group within the Chytridiomycota phylum (Figure 15). The three OTUs present high identity level (>98%) with environmental sequences ,and form a monophyletic group whose closest described relative is a sequence from the genus *Maunachytrium*. These OTUs constitute a new clade in the Lobulomycetaceae family (Simmons *et al.*, 2009). BLASTn searches of these environmental sequences return the *Maunachytrium* sequence as the best hit, with a maximal identity of 96%. The widely used BLAST-based annotation for environmental sequences, would end with an assignation to *Maunachytrium keaense* or *Maunachytrium sp*. However, by choosing a phylogenetic approach, the analysis goes into greater depth.

The initial positioning of these sequences suggests that they form a new clade within the Lobulomycetaceae family, outside the *Maunachytrium, Lobulomyces* (maximal identity 93%) and *Clydaea* (maximal identity 92%) genera.

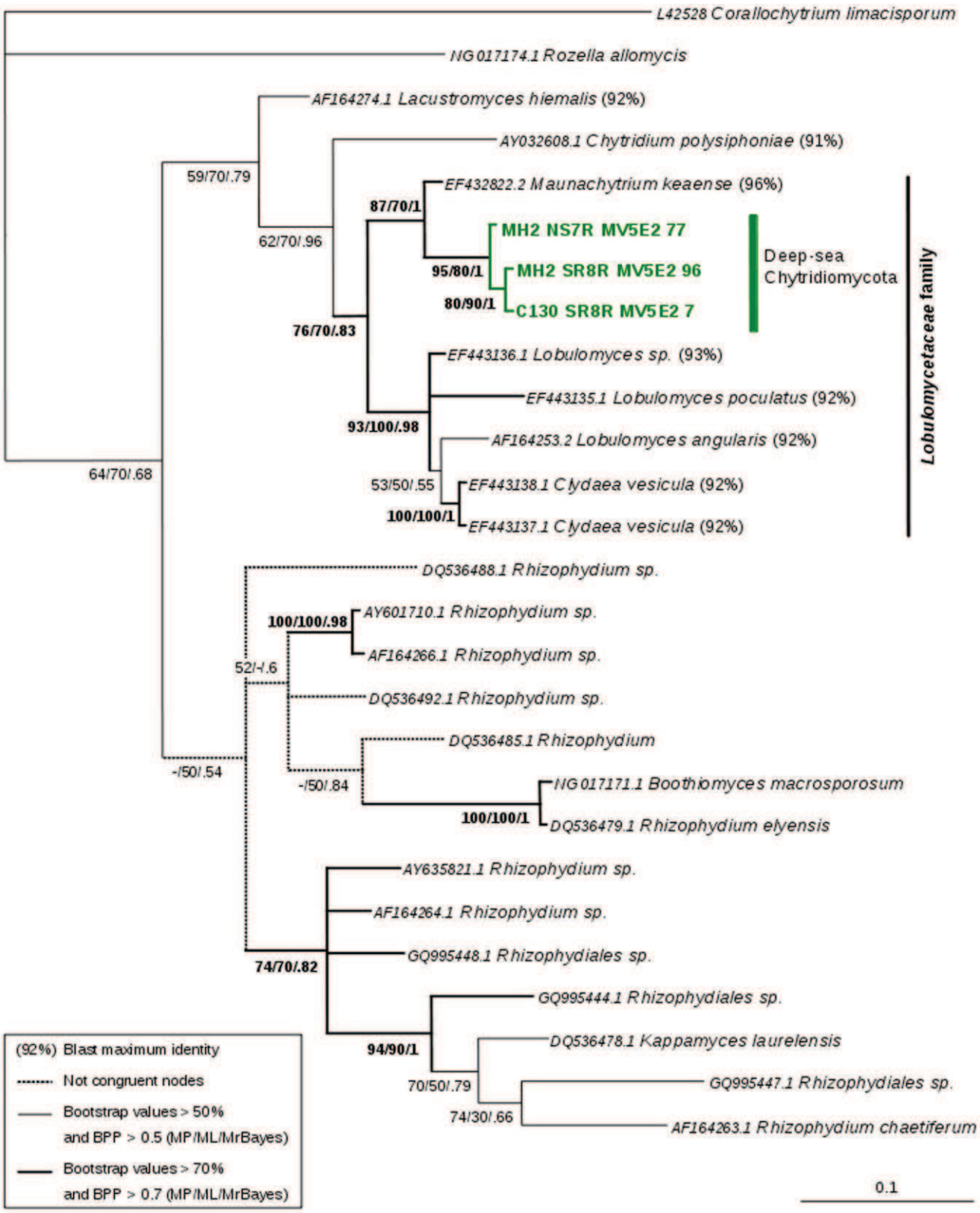


Figure 15 :

SSU rRNA phylogenetic positions of deep-sea Chytridiomycota (colored terminals) along with the closest known related SSU rRNA fungal sequences.Topology was built using MrBayes v.3.1.2 (Scale bar: 0.1 estimated substitutions per site, 3000000 generations sampled every 100 generations and an average standart deviation of split frequencies of 0.004140) from a ClustalW 2.1 alignment. The model GTR+I+G was designated by jModelTest 0.1. Node support values are given in the following order: Maximum Parsimony/Maximum Likelihood (both calculated with PAUP 4.0β10 version, 500 bootstraps)/MrBayes. *Corallochytrium limacisporum* (L42528), a putative choanoflagellate, was used as outgroup. *Maunachytrium keaense* (it is not part of PHYMYCO-DB) was also used to help build the tree. All sequences are listed with their GenBank accession numbers. The topologies were congruent apart from doted lines indicated in the figure. Thin lines show bootstrap values >50% and BPP >0.5 (MP/ML/MrBayes) and thick lines: bootstrap values >70% and BPP >0.7 (MP/ML/MrBayes). The sequences belonging to the Lobulomycetaceae family are indicated with their BLASTn percentage of maximum identity compared to the three deep-sea Chytridiomycota OTUs.

This exercise thus highlights important differences between phylogeneticaly based annotation and BLASTn annotation. More and more identifications rely solely on BLAST searches which allow for faster analyses of the rapidly increasing numbers of environmental sequences. Indeed many analyses and tools developed for mass sequencing are based on BLAST searches (e.g. MEGAN). We would argue that this approach is less conservative and more prone to mistakes. The use of phylogenetic approaches, when it is possible should be favoured, to avoid increasing the presence of polluting sequences in international sequences databases.

Discussion

The release of PHYMYCO-DB is expected to provide comprehensive access to fungal sequences for two phylogenetic markers (SSU rRNA and EF1-α genes) obtained from cultivated isolates, as well as environmental samples. As a result of deep sequence cleaning, the aligned sequences available in PHYMYCO-DB are of high quality (Figure 13). To our knowledge, this curation strategy provides a novel approach to the problem of database pollution. As such, we anticipate that it will complement other existing databases such as the “Assembling the Fungal Tree Of Life” project (AFTOL ; Lutzoni *et al.* , 2004), UNITE (Kõljalg *et al.*, 2005 ; Abarenkov *et al.*, 2010a) and MaarjAM (Öpik *et al.*, 2010)

which are restricted to fungal sequences.

Curation and annotation of ITS is made possible through the web-based-workbench of PlutoF (Abarenkov *et al.*, 2010b). Initially, the UNITE system contained ITS and nLSU/28S rRNA gene sequences from Basidiomycota and Ascomycota. Based on recent work, the ITS region is now being suggested as a possible universal DNA barcode marker for fungi (Schoch *et al.*, 2012). It is accepted that the ITS region is valuable at species level and so, more taxonomically informative than SSU rRNA gene sequences for analysing groups of organisms that have emerged 'recently' and are closely related (Anderson & Parkin, 2007), e.g. Ascomycota and Basidiomycota. The ITS region is also often used to resolve phylogenetic relationships at the species level or at the infraspecific level (Xu *et al.*, 2007). However, as the ITS region displays high sequence variability, even within a given organism as in Glomeromycota (i.e. Sanders *et al.,* 1995), obtaining reliable alignments with this marker can be difficult (D'Auria *et al.*, 2006) and potentially precludes multiple alignments. This is because accurate comparisons are hindered by the accumulated homoplasy and the high frequency of insertion/deletion events. The use of the SSU rRNA sequences is interesting since new groups, within all the fungal phyla including Ascomycota and Basidiomycota, can be detected (i.e. Vandenkoornhuyse *et al.,* 2002a ; Bass *et al.*, 2007). The MaarjAM database has focused on SSU rRNA gene of arbuscular mycorrhizal fungi (Glomeromycota), with associated metadata. The existence of this database and the potential emergence of others should be encouraged. It enables the community to have access to reliable sequences.

For fungal sequence annotations and phylogenetic interpretations of fungal environmental sequences, one of the main advantages of PHYMYCO-DB is to facilitate the primer design and subsequent phylogenetic analyses of amplicons as shown in the example above (Figure 15). The use of PHYMYCO-DB to perform expert analyses appears to be complementary to BLASTn, the latter allowing a quick look of the query sequence proximity compared to the available sequences. From the phylogenetic analyses performed one arising interpretation is that different apparent polyphyletic groups may be a consequence of wrong annotations. We anticipate that the use of PHYMYCO-DB will help to limit incorrect SSU rRNA and EF1-α genes fungal annotation propagation in sequence databases.

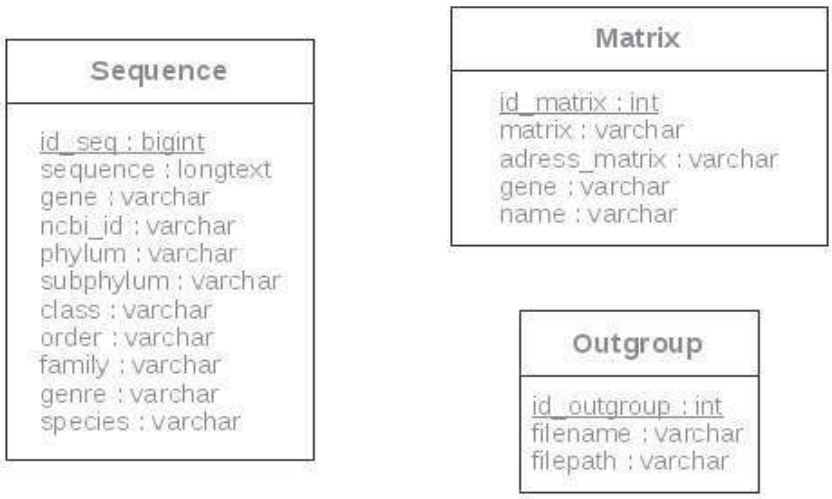
Availability and Future Directions

The PHYMYCO-DB is available via a web-based interface at http://phymycodb.genouest.org/ on the GenOuest bioinformatics platform web site. The web interface is divided into 2 parts. The first part, entitled “DB admin”, is restricted to the administrators for use in cleaning and optimising the database. The second part, entitled “DB explore”, is publicly accessible to all users. The next set of PHYMYCO-DB releases will include (i) the provision of alignment files in which outlier nucleotides identified from influence functions (Bar-Hen*et al.*, 2008) will be highlighted, so that users can then delete these sites (ii) taxonomic modifications within Chytridiomycota and Zygomycota after Hibbett *et al.* (2007) and after Jones *et al.* (2011). PHYMYCO-DB will continue to expand with new genes. We are currently investigating β-tubulin (tub1, tub2), actin (act1), and RNA polymerase II subunits (rpb1 and rpb2) as potential interesting targets. PHYMYCO-DB will also be improved by incorporating all the finished fungal genomes available, and increasing the diversity of tools to perform multiple alignments.

Supporting Information

MySQL table structure of PHYMYCO-DB.

Figure S11 :



Acknowledgments

We thank the GenOuest Bioinformatics core facility and anonymous referees for valuable comments on the manuscript.

Author Contributions

Conceived and designed the experiments: PV AD OC. Performed the experiments: SM MD. Analyzed the data: SM MD TLC. Contributed reagents/materials/analysis tools: AD OC LG LS AB ETK. Wrote the paper: PV SM MD ETK. Creation and design of the database: OC LG LS AB. Extraction of data from Genbank: OC LG LS AB.

Chapter IV:

Does a decrease in plant diversity lead to a decrease in AM symbiont diversity?

Introduction

Mutualisms, cooperative interactions between different species, are an important driver of ecosystem dynamics. In particular, the mutualism between plants and their arbuscular mycorrhizal (AM) fungi is responsible for massive nutrient transfer and storage. In exchange for up to 20% of the total photosynthesized carbon, AM fungi provide plants with nutrients (i.e. as P, N, oligoelements) (Smith & Read, 2008; Fitter *et al.* 2011). Plants are thought to have successfully colonized land thanks to the mutualist association with arbuscular mycorrhiza (Simon *et al.*, 1993; Redecker *et al.*, 2000). Current estimates suggest that AM fungi colonize 80% of land plants.

As developed in the introductory section, AM fungi can have multiple effects on individual plants. As a consequence, they are also important drivers of plant community dynamics. Experimental work suggests that a higher AM fungal diversity positively influences plant productivity, diversity and nutrient uptake (van der Heijden *et al*. 1998; Hooper *et al.,* 2005 ; Maherali & Klironomos, 2007; Wagg *et al*., 2011a). This is consistent with Moora *et al*. (2004) observations showing that different AM fungal taxa induce different growth responses depending on the host plant. AM fungal diversity likely contributes to plants coexistence (Grime *et al*., 1987; Van der Heijden *et al.,* 2003) and may relax plant-plant competition (Wagg *et al.,* 2011b).

Reciprocally, plant communities are also able to influence belowground AM fungal communities (Johnson *et al*. 2004). However, host plant effects on AM fungal diversity have yet to be studied extensively; only a limited number of studies have been published up to date (e.g. Verbruggen *et al*., 2010; Hartmann *et al*., 2009; Martinez-Garcia *et al.,* 2011). For example, the plant neighborhood effect (i.e. effects of the surrounding plants of the host plants on its arbuscular mycorrhizal fungal community) on AM fungal diversity has been poorly investigated. To better understand plant effects on fungal communities, a spatialized sampling strategy and knowledge of plant community history is needed.

One idea is that a higher plant diversity is linked to a higher variety of carbohydrate substrates (Tilman *et al.*, 1992; 1996a) which in turn increases the decomposers diversity

and functional complementarity. If we assume that plant growth is limited by resources availability, higher nutrients are available when organic matter is better recycled, thus when the plant diversity is higher. Plant diversity and ecosystem productivity are positively correlated (Tilman *et al.*, 1996b; 2001; Hector *et al.*, 2000). However, this positive correlation between plant diversity and productivity implies a range of AM fungal functions (i.e. plants nutrition, water supply), and that individual plant requirements are harmonized with particular AM fungal functions. As shown by Vandenkoornhuyse *et al.* (2002b; 2003) a host-plant preference in AM fungi does exist for co-occuring plant species. This can be interpreted as a consequence of host-plant requirements and the ability of the AM fungi to complement these needs. In light of these observations, the loss of fungal symbionts diversity could lead to a degradation in ecosystem nutrient cycling and to a negative impact on soil fertility. Considering this host plant preference, we hypothesize that a decrease in host-plant diversity will lead to a decrease in fungal symbiont diversity (see also the 'General introduction' section).

Here we address this hypothesis and try to understand how plant diversity and composition affects root fungal communities. To achieve this, we collected samples from plots of a long term experiment where the plant diversity has been manipulated (Figure 16; Cedar Creek biodiversity experiment, Univ Minnesota, USA). Samples are being analysed using emerging molecular techniques allowing for the characterization of fungal communities. Five sampling campaigns have been run to assess both inter-annual and seasonal changes of root associated fungal communities along a gradient of plant species richness in 51 different plots.

As the analyses are still ongoing, this chapter provides preliminary results obtained from mass sequencing of AM fungal diversity in roots collected in June 2011 (Table 2). However, these preliminary results offer new perspectives about the possible consequences of the plant diversity erosion on the consortium of root fungal inhabitants. These results can also be interpreted in light of current conventional agricultural practices.

Material and methods

Sampling

In order to examine the effects of host plant species richness on AM fungal community composition and diversity, we used the biodiversity experimental plots of the Cedar Creek LTER (i.e. Long Term Ecological Research) (see figure S12in the supplementary material) (Minnesota, 45°35′N, 93°10′W) established in 1994 by David Tilman to follow the effect of plant diversity variations on ecosystem functioning. The experimental area occupies a 10 hectare block of land and contains a total of 342 plots with a plant species gradient of diversity. The LTER area was first treated with herbicide and burned in 1993. Then 8 cm of the soil were removed to reduce the presence of seeds. All plots were manually seeded a first time with a total of 10g/m2 of seed in 1994 and a second time with a total of 5g/m2 of seed in 1995, and they were watered. Each plot measures 13m*13m, only the central 9*9m are used for the sampling to avoid an edge effect. Plots are actively maintained and manually weeded throughout the years to preserve the specified species and level of plant diversity. The plant richness gradient goes from one plant species to 32 plant species. All the 5 sampling campaigns were carried out on the e120 experiment (Biodiversity II), which contains a subgroup of 168 plots of monocultures (39 replicates), 2 plant species (35 replicates), 4 plant species (29 replicates), 8 plant species (30 replicates) and 16 plant species (35 replicates). Plants functional groups are forbs, C3, C4, and legumes (for details, see http://www.cbs.umn.edu/cedarcreek). The plant species composition within each plot was chosen randomly from a pool of 18 grassland perennial species (Table 1)

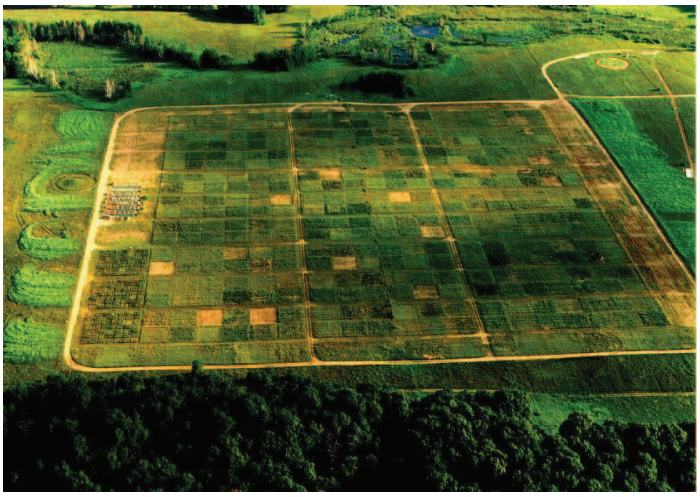


Figure 16:
Cedar Creek biodiversity experiment, Univ Minnesota, USA

Species	Abbreviation	Functional Group
Achillea millefolium	Achmi	Forb
Agropyron smithii	Agrsm	C-3
Amorpha canescens	Amoca	Legume
Andropogon gerardi	Andge	C-4
Asclepias tuberosa	Asctu	Forb
Elymus canadensis	Elyca	C-3
Koeleria cristata	Koecr	C-3
Lespedeza capitata	Lesca	Legume
Liatris aspera	Liias	Forb
Lupinus perennis	Luppe	Legume
Monarda fistulosa	Monfi	Forb
Panicum virgatum	Panvi	C-4
Petalostemum candidum	Petca	Legume
Petalostemum purpureum	Petpu	Legume
Petalostemum villosum	Petvi	Legume
Poa pratensis	Poapr	C-3
Schizachyrium scoparium	Schisc	C-4
Solidago rigida	Solri	Forb
Sorghastrum nutans	Sornu	C-4

Table 1:
Plant species present in the e120 experiment. In this table 20 plant species are listed and not 18 because when *Petalostemum villosum* was planted, the seeds also contained a congener contaminant plant species: *P. candidum.* Thus in plots containing *P. villosum,* both species were planted. In addition, when the LTER was set up, no *Solidago rigida* were able to grow. This species was replaced by *Monarda fistulosa*. However *S. rigida* established one year later causing both plants to be present.

Five sampling campaigns were done: early June 2011, end of May 2012, beginning of July 2012, beginning of September 2012 and mid May 2013. In this chapter we present the results obtained from samples of the first sampling campaign. The same plots were harvested for each subsequent sampling campaign.

The choice of the plots to harvest was based on the function of the plants it contained. The plants considered were chosen on the basis of their good establishment and maintenance within the biodiversity experiment : *Andropogon gerardi, Schizachyrium scoparium, Poa pratensis, Lespedeza capitata, Liatris aspera, Lupinus perennis, and Koeleria cristata.* Thus, a total of 51 plots were selected, 12 plots containing 1 plant species, 12 containing 2 species, 10 with 4 species, 12 with 8 species and 5 with 16 species (Table 2).

1 species		2 species		4 species		8 species		16 species	
5	Andge	157	Agrsm Koecr	138	Achmi Elyca Koecr Liaas	130	Achmi Asctu Elyca Liaas Panvi Petpu Quema Schsc	202	chmi Agrsm Amoca Andge Asctu Elyca Koecr Lesca Liaas Luppe Monfi Panvi Petpu Poapr Schsc Solri Sornu
29	Lesca	168	Andge, Koecr	176	Agrsm Liaas Panvi Poapr	170	Achmi Asctu Elyca Koecr Monfi Petca Petpu Petvi Queel Solri	227	Agrsm Amoca Andge Asctu Elyca Koecr Lesca Liaas Luppe Monfi Panvi Petpu Poapr Quema Schsc Solri Sornu
83	Luppe	171	Koecr Luppe	201	Lesca Petpu Queel Sornu	177	Andge Asctu Koecr Liaas Petca Petvi Quema Schsc Sornu	253	Achmi Agrsm Amoca Andge Asctu Koecr Lesca Liaas Luppe Monfi Panvi Petpu Poapr Quema Schsc Solri Sornu
94	Lesca	175	Luppe Sornu	223	Koecr Liaas Poapr Queel	178	Achmi Agrsm Elyca Koecr Liaas Monfi Panvi Schsc Solri	273	Achmi Andge Asctu Elyca Koecr Lesca Liaas Luppe Monfi Panvi Petpu Poapr Queel Quema Schsc Solri Sornu
109	Andge	193	Andge Luppe	225	Elyca Petpu Queel Schsc	206	Agrsm Andge Asctu Lesca Luppe Monfi Poapr Solri Sornu	339	Achmi Amoca Andge Asctu Elyca Koecr Lesca Liaas Luppe Monfi Panvi Petpu Poapr Queel Quema Schsc Solri
135	Schsc	236	Lesca Panvi	229	Andge Petpu Poapr Schsc	208	Achmi Agrsm Koecr Lesca Luppe Panvi Poapr Schsc	plots	
142	Koecr	259	Lesca Schsc	233	Liaas Petpu Poapr Queel	210	Achmi Elyca Koecr Lesca Liaas Luppe Poapr Schsc		
167	Liaas	300	Luppe Panvi	286	Lesca Poapr Schsc Sornu	213	Achmi Andge Koecr Lesca Petca Petvi Poapr Quema Schsc		
237	Poapr	304	Agrsm Koecr	302	Liaas Luppe Monfi Quema Solri	232	Koecr Luppe Monfi Panvi Petca Petvi Queel Schsc Solri Sornu		
265	Luppe	311	Koecr Panvi	325	Koecr Monfi Panvi Petpu Solr	292	Andge Elyca Koecr Lesca Luppe Petca Petvi Poapr Sornu		
267	Liaas	330	Andge, Liaas			303	Achmi Agrsm Koecr Liaas Luppe Monfi Petca Petvi Poapr Solri		
268	Koecr	342	Luppe Monfi Solri			313	Achmi Koecr Lesca Liaas Luppe Petca Petpu Petvi Quema		
12 plots		12 plots		10 plots		12 plots			

Table 2:
List of the plots sampled and related plant species richness. Numbers on the right for each modalities are the plot identifiers.

Roots harvesting

Five 6X18cm soil cores were sampled in each plot (figure 17A). Within the next 24 hours, roots were separated from the 255 core samples by sieving (figure 17B). Three soil

aliquots were taken and frozen and roots were washed in tap water then with a 1% solution (V/V) of TRITON X100 and finally rinsed with tap water and distilled water. Cleaned roots were selected (figure 17C) Roots were frozen in 5 different eppendorf microtubes (figure 17D). All the samples were stored at -80°C until utilisation.

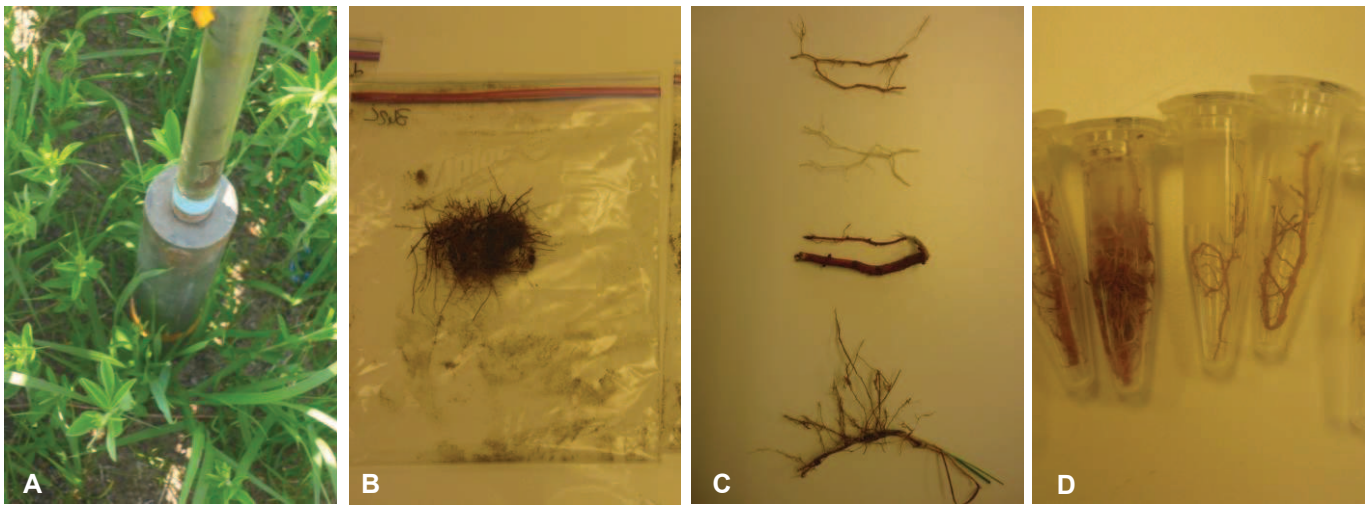


Figure 17:
Steps of the roots sampling: A), soil cores are done, B) roots are separated from soil, C) roots are selected, and D) put in eppendorf tubes.

DNA extractions

Root samples were ground to powder in liquid nitrogen using a micropestle. Total DNA was extracted using a DNeasy plant mini kit (Qiagen Ltd, Crawley, UK) following the manufacturer's instructions. The DNA obtained was eluted in a final volume of 150µl.

Leaves and roots identification

Leaves from each of the 18 plant species present in the sampled plots were taken so that the roots present in each samples could be identified by analysing the chloroplastic *trnL* intron. This marker is used as a molecular barecode for plant species identification. DNA was extracted from each leave sample with DNeasy plant mini kits (QiagenLtd, Crawley, UK). The *trnL* intron was amplified from these DNA templatesby PCR using the primers 'c' (5' CGAAATCGGTAGACGCTACG 3') and 'd' (5'GGGGATAGAGGGACTTGAAC 3') (Taberlet *et al,* 1991). The size polymorphism of the amplified fragment makes it easy to

identify plants based on leaf DNA. The PCR was performed using illustra Ready To Go PCR Beads (GE Healthcare, UK) with a mixture of 6.25 pmol of each primer and 5µl of DNA template in a final volume of 25µl. The PCR cycling consisted of : a first denaturation step at 94° for 3min, followed by a denaturation step at 94° for 45 sec, a hybridization step at 58°C for 1min and an elongation step at 72° for 1min. These three steps were repeated 35 times and the cycling regime was ended by a final elongation step at 72° for 5 min.

Amplifications

A fragment of the 18S rRNA gene of the AM fungi was specifically amplified from the total DNA extracted from roots. The primers used were AM1 (Helgason *et al.,* 1998) and NS31 (Simon *et al.,* 1992). This primer set allowed a PCR amplification of a 550bp DNA fragments. The amplicons were sequenced with a 454GSFLX sequencer (Roche). Fusion primers were designed to allow the multiplexing. AM1 was bound to different multiplex identifiers (MID hereafter) to identify the amplicon origin within the sequences produced. A MID consists of a particular sequence of 6 to 10 nucleotides acting like a sample barcode. In this study we designed 96 original MIDs to allow the multiplexing of amplicons from 96 different samples. The sequence constituted by the AM1 primer and the MID was followed by an adapter A (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG 3'). Each fusion primer (Figure 18) was designed in a way to avoid strong secondary structures and to prevent formation of self-dimers and hetero-dimers. For this purpose, the ΔG of each candidate primer was calculated using RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). ΔG values > -10 were accepted. The GC% of each primer was kept between 40 and 60%. The primer NS31 was bound to the adapter B (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG 3') to fit with the lib L sequencing chemistry (for more details see the part *Sequencing 454,* Figure 18).

All the PCRs were performed using illustra Ready To Go PCR Beads (GE Healthcare, UK) with a mixture of 6.25 pmol of each primer and 5µl of DNA template in a final volume of 25µl. PCR conditions were optimized to have no primer dimers and specific products. The different amplification steps were (i) a first denaturation step at 94° for 4min, (ii) a denaturation step at 94° for 30 sec, (iii) an hybridization step at 62°C - 0.1°C per cycle for 45 sec (iv) an elongation step at 72°C for 1 min. These last 3 steps were repeated 35 times. The cycling regime ended with a final elongation step at 72° for 7 min.

The quality of the amplifications was checked using a 1% E-Gel® (Life Technologies-Invitrogen). A PCR and a true PCR replicate were performed for each DNA

sample. All PCR products were purified using AMPure XP – PCR kit (Roche) to prepare amplicon libraries. The quality of the purified product was checked using Agilent High Sensitive DNA assay (Agilent Technologies). The concentrations of all the purified amplicons were then measured by dPCR using a Fluidigm EP1 instrument. The amplicons were then pooled all together.

emPCR

The pooled amplicons were amplified by emPCR using the Lib L chemistry. First, amplicons were mixed with micro beads in excess. An emulsion leads to the formation of millions of nanoreactors. Some of these nanoreactors contain only one PCR bead linked to a single amplicon. The emPCR amplification cycle is consists of (i) a first denaturation step at 94° for 10min, followed by (ii) a denaturation step at 94° for 1min, (iii) an hybridization step at 60°C for 1min and (iv) an elongation step at 72° for 1min. These last three steps were repeated 35 times and followed by a final elongation step at 72° for 7 min.

Sequencing 454

Sequencing was performed using the GS FLX sequencer (Roche/454). The Lib L library was chosen to sequence the amplicons unidirectionally. Amplicons of the 2 true PCR replicates were sequenced in parallel. The amplicons library and sequencing were replicated to ensure that the sequences found in the samples were real sequences and not stemming from PCR or sequencing errors. True sequences are expected to display a 100% homology level. Thus our sequence analysis pipeline calculated the pairwise homology level to constitute groups of identical sequences. Sequences found only in one of the two replicates were not considered as valid, and were removed from the OTU list.

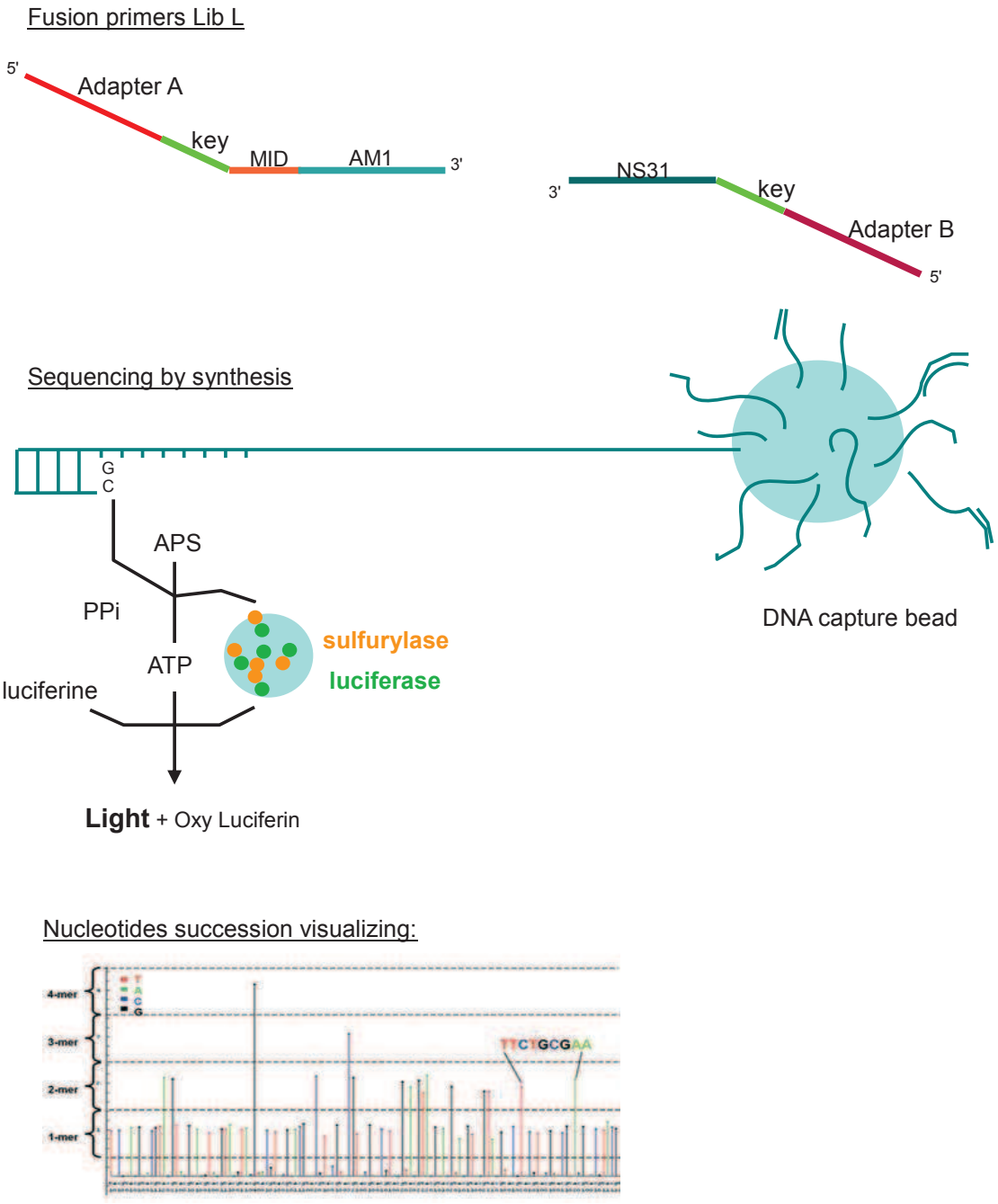


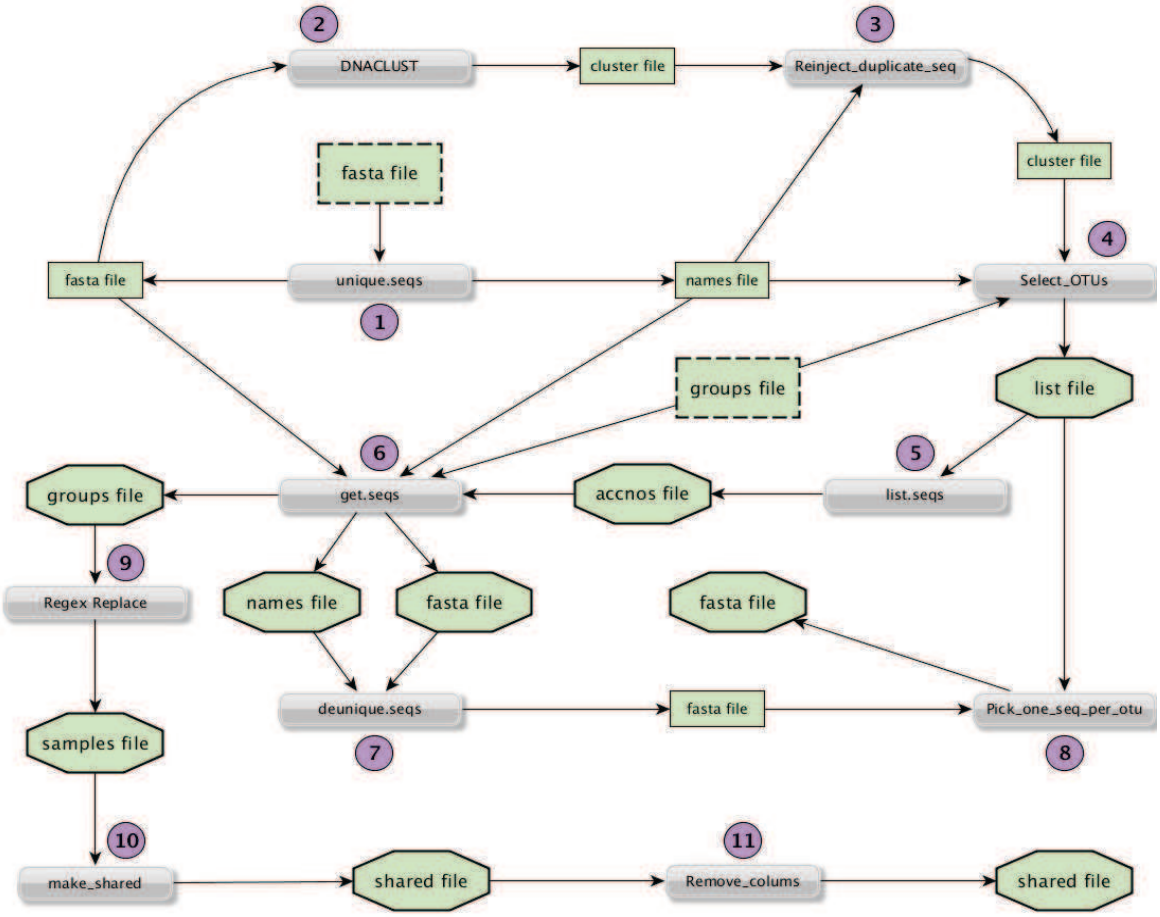
Figure 18:

Principle of 454 GS FLX sequencing. The two Lib L adapters allow DNA capture by the beads (adapter B) and the beginning of sequencing (adapter A). The sequencing is done by synthesis as explained in the figure. When a nucleotide is incorporated a light signal is emitted and detected by a CCD camera. Pictures are recorded and then converted into sequences.

Analyses pipeline

A workflow was created for efficient processing the data analyses. This workflow

processes the data in a combined succession of operations within a Galaxy environment (http://genosites.genouest.org/) and assembles the sequences in clusters to form OTUs. The sequences are first clustered with a threshold of 97% identity.



Legend:

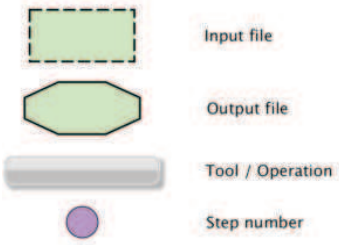


Figure 19:
This figure shows the details of the amplicon-sequence analysis pipeline to obtain OTU from a .sff dataset obtained after 454GSflx+ pyrosequencing. Pairwise distances between sequences are calculated to determine the different OTUs within the dataset and to assess the species richness and evenness (cutoff d<0.97). Sequences are clustered depending on their similarities and compared to a chosen RNA/DNA database (RDP, GreenGenes, or Phymyco-DB) to define their taxonomy.

A file containing the sequences to be processed and a 'group file' relative to the sequence file are required to start the analyses (figure 19). Once these files have been uploaded the workflow can be launched. Different files are automatically created during the analyses and are made available to the user. These files are (1) a list file compiling the different OTUs obtained, (2) a fasta file listing the sequences contained in OTUs, (3) an accnos file relative to the fasta file (containing a list of the sequence IDs), (4) a name file relative to the fasta file, (5) a samples file relative to the fasta file, (6) a groups file relative to the fasta file, (7) a fasta file with one representative sequence of each OTU, (8) a shared file linking OTUs, samples and sequences identity along with the number of sequences from the sample contained in that OTU (i.e. a contingency table), and finally, (10) a contingency table for each OTU within each sample (see figure 19; Bahin *et al.,* 2013, in prep.)

Phymyco-DB (Mahé *et al.*, 2012) is a database that was developed to facilitate fungal sequence analyses (details in Chapter III). This database was propagated into the workflow to allow identification of the different OTUs obtained.

Statistical and phylogenic analysis

The identity and diversity of AM fungal OTUs present in the harvested roots was assessed by processing a phylogenetic reconstruction. Sequences of the different OTUs were aligned with ClustalX 2.0.10 (http://www.clustal.org/ ; Larkin *et al.,* 2007). A phylogenetic tree based on maximum likelihood was constructed using PhyML 3.0 aLRT (Guindon *et al.*, 2010) and bayesian inferences with MrBayes v.3.1.2. (Ronquist *et al.,* 2011) The model to apply was determined using jModelTest0,1 (Posada & Crandall, 1998).

The relationships between AM fungal species richness and plant species richness were examined by performing an FCA using R (http://www.r-project.org/).

Concordance index analyses were also performed using R to test each OTU for the possibe existence of a rule of assemblage related to the plant species level modalities. The concordance index is calculated using the formula $S = (a+d) / (a+b+c+d)$ where a= number of OTUs within 2 common modalities, b and c = number of OTUs absent from one of the 2 modalities, d=number of OTUs absent at the same time within 2 modalities. From this, a pairwise concordance index among modalities is computed. A dendrogram grouping those OTUs with concording responses across modalities is deduced.

The significance of the differences between groups of OTUs was determined with a

Kruskal Wallis test followed by a Nemenyi post-hoc test. The differences between the OTU richness as a function of the plant richness were assessed after checking the homoscedasticity of the variances with a Bartlett test and the normality of the data by a shapiro test. Then, an ANOVA was carried out followed by a Tukey HSD post-hoc test.

Preliminary results and discussion

A deep multiplexing was completed within the pyrosequencing runs by using fusion primers and a mixture of amplicons obtained for a given molecular target from different samples. Sequences from amplicons of AM fungi were analysed thereafter, while sequence amplicons of the whole fungal pool associated with roots were removed from the dataset because the coverage (i.e. number of sequences for a given amplicon) was too low to describe the whole fungal diversity. In total, slightly more than 300,000 filtered AM fungal sequences were analysed.

The AM fungal community structure in relation to plant species richness modalities was analysed by ordination and clustering methods (figure 20). These analyses suggest a change in AM fungal communities for the 4 and 8 plant species plots. In contrast, a high variance in the AM fungal community was observed for the samples fromplots with 1 and 2 plants species. One possible interpretation is that the single plant species plots are contaminated by other plant species despite the intensive work performed to weed and maintain the plots. This implies that plots containing one or more plant species were in fact analysed. The AM fungal community of the plots with 8 plant species was distinct from that of the other modalities as the samples from this modality are similar in the OTUs they contain (figure 20). It seems that the greater the plant species richness is, the higher and the more convergent the AM fungal community diversity is (figure 20). We thus hypothesize that if a host-plant preference exists, when plant species richness is low, the AM symbionts associated with these plants will preferentially develop and spread. We expected that a low plant species richness would result in a low AM fungal diversity and spread. In contrast with a high plant species richness, the AM fungal diversity theoretically possible will also be higher. This is predicted to lead to the colonization of the roots of surrounding plants, leading to an homogenization of the AM fungal community present in roots (i.e. even if samples from the 4 & 8 plant species modalities display a higher OTU richness, AM fungal community composition found in the samples of these modalities looks like each other).

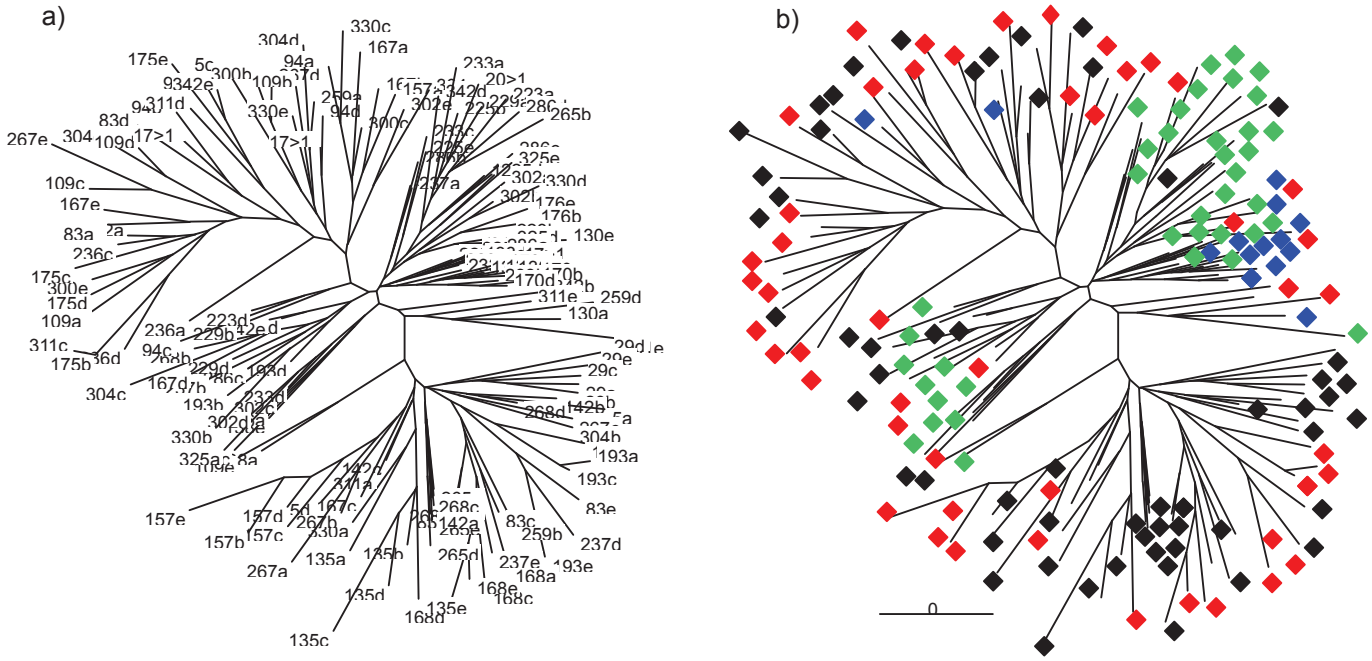


Figure 20:

AM fungal communities clustering for each sample analysed. This clustering was calculated using ClustalW 2.0.10 using a presence/absence matrix. It results in an unrooted tree where samples close to each other have a similar OTU composition (similarity in OTU number and identity). The AM fungal communities in black, red, green and blue are respectively from plots containing 1, 2, 4 and 8 plant species.

The diversity of AM fungal OTUs colonizing roots of the plants sampled in Cedar Creek were analysed using a phylogenetic reconstruction. From a total of 304,690 filtered sequences a total of 54 OTUs all belonging to the Glomeromycota were highlighted. OTUs representative of 4 orders of the Glomeromycota phylum were found (Figure 21). Glomeraceae were dominant with approximately 90% of the total number of sequences belonging to this group. Diversisporales, Paraglomerales, and Archaeosporales accounted respectively for 5 %, 1% and 0.1 % of the total number of phylotype sequences. Glomeraceae is the most represented group with 38 OTUs (i.e. 73% of the total number of sequences) whereas the Diversisporales contain 9 OTUs (17 %). The number of OTUs found is in the comparable to other studies using a similar sequencing strategy. Opik *et al.* (2009) found a total number of 47 OTUs, compared to 32 OTUs for Lekberg *et al.* (2012)

and 70 for Lin XJ *et al.* (2012). The resulting phylogenetic tree is in accordance with the topologies described in literature with a large majority of Glomerales followed by Diversisporales, Archaeosporales and Paraglomerales (Lekberg *et al.*, 2012; Lin XJ *et al.,* 2012).

Interestingly most of the OTUs found do not have any close sequence relative. The majority of the OTUs presented in figure 21 are unknown. The results also suggest a gap between the AM fungal taxonomy / species description and the AM fungal species diversity in nature. AM fungal diversity is far from being adequately described. From our results, we can speculate that a large proportion of the AM fungal species diversity remains unknown. Again, this is in agreement with Opik *et al.* (2009). This phenomenon was reinforced by the fact that we used a deep sequencing strategy which enabled us to detect OTUs at very low abundances. We have noticed that OTU 63 which is an Archaeoesporales has an Ascomycota as best BLAST hit, which is an example of the possible propagation of an incorrect annotation if a phylogeny is not constructed.

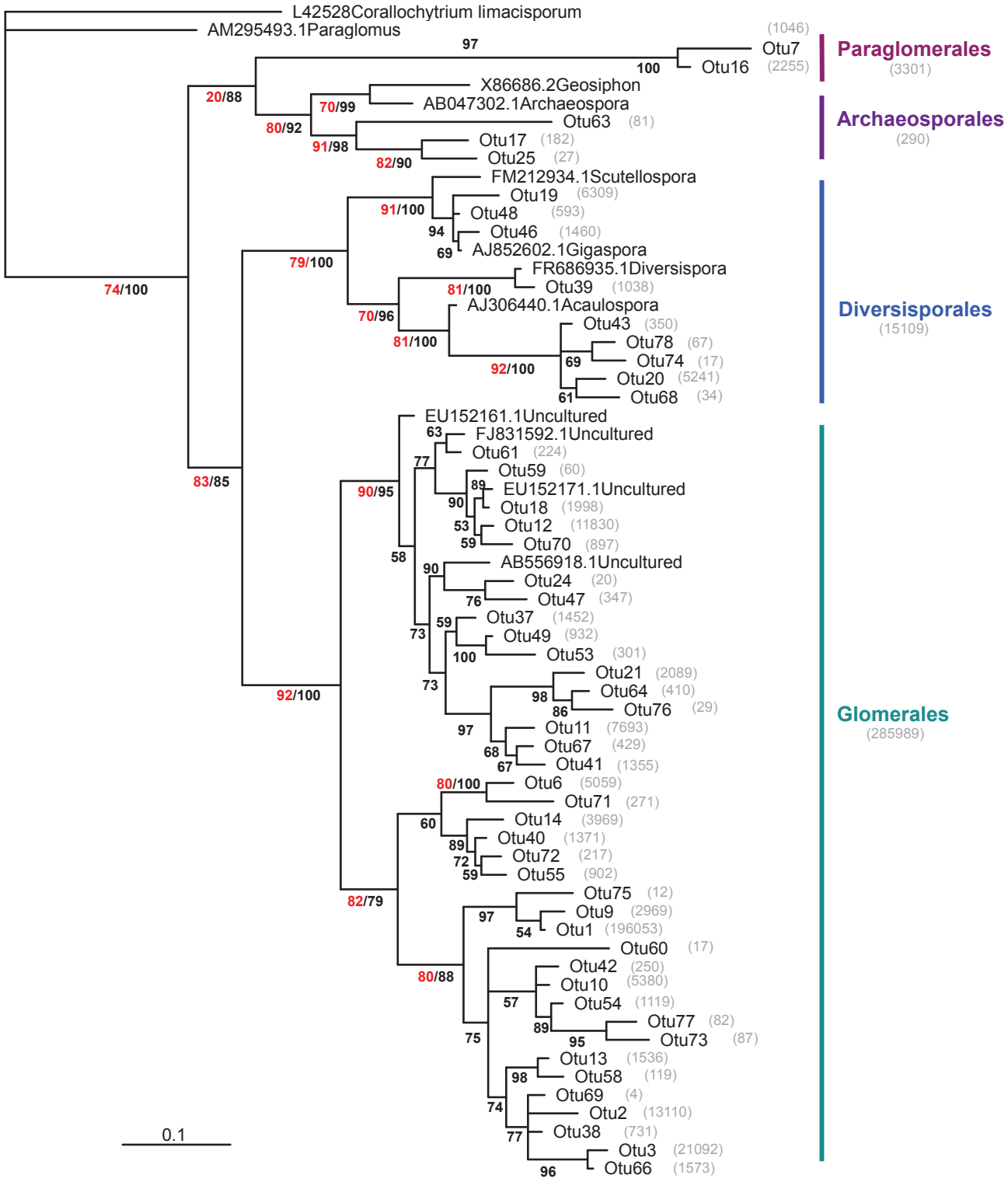


Figure 21:

Phylogenetic analysis of the SSU rRNA AM fungal OTU representative sequences (i.e. one sequence per OTU found; one OTU being defined by a 97% cutoff). The phylogeny was built by bayesian inferences using MrBayes v.3.1.2 (Scale bar: 0.1 estimated substitutions per site, 3300000 generations sampled every 100 generations and an average standart deviation of split frequencies of 0,00958) from a ClustalW 2.1

alignment. The GTR+I+G model was selected after a ModelTest analysis. Node support values are given in the following order: in red, Maximum Likelihood (calculated with PhyML 3.0 aLRT, gamma shape parameter: 0.392, number of categories: 4, proportion of invariant: 0.117)/ in black, MrBayes. *Corallochytrium limacisporum* (L42528), a putative choanoflagellate, was used as outgroup. All sequences are listed with their GenBank accession numbers. OTUs 4, 5, 50, 51, 52 and 79 were removed from the phylogenetic reconstruction because they induced noise in the sequence alignment. Their closest relative sequences are respectively: uncultured *Glomus* (HF566605.1), uncultured *Glomus* (HF568342.1), uncultured *Glomus* (KC589000.1) uncultured *Glomus* (HF566497.1), uncultured fungus (HE806403.1) and uncultured *Glomus* (HF913471.1).

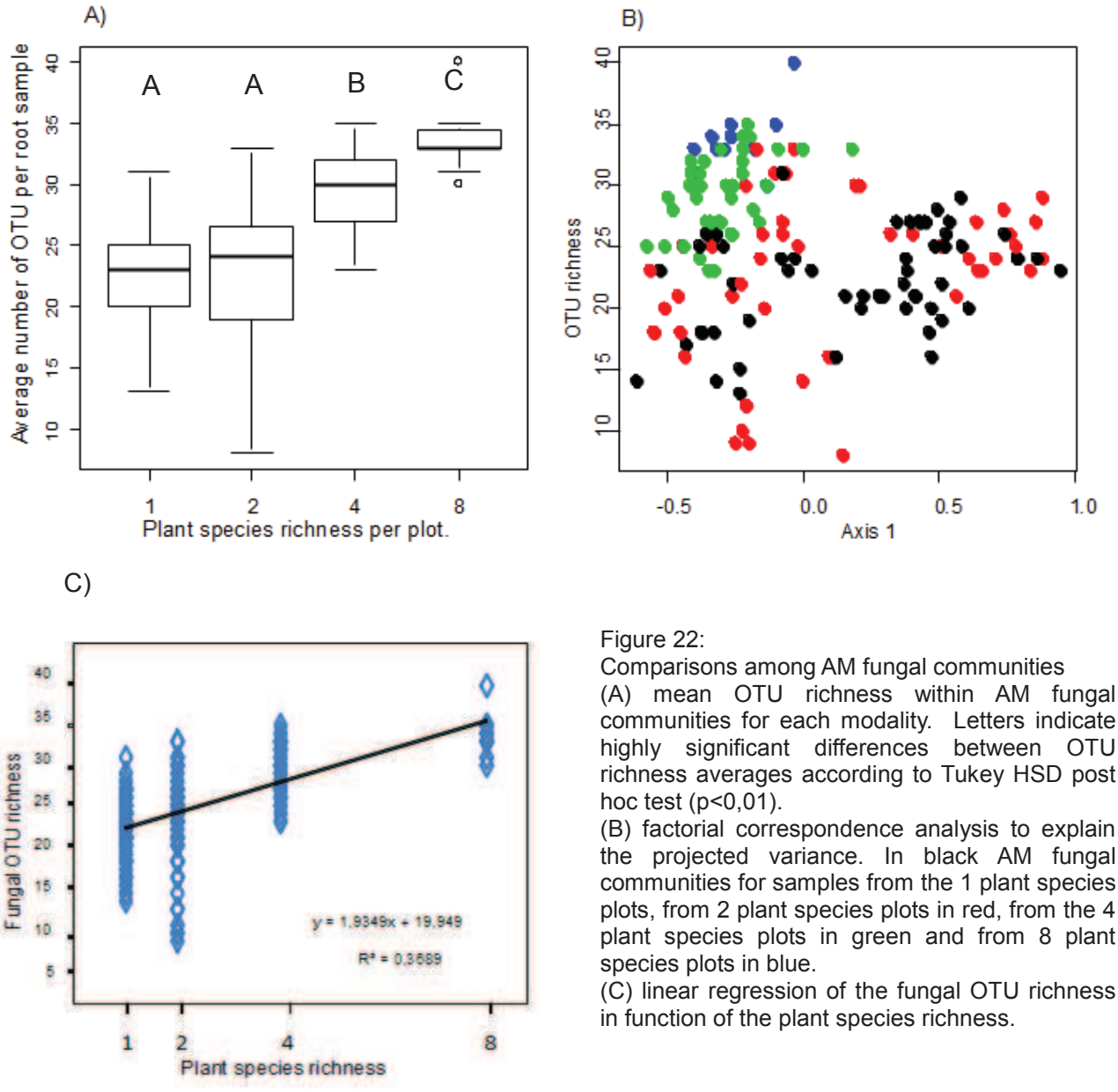


Figure 22:
Comparisons among AM fungal communities
(A) mean OTU richness within AM fungal communities for each modality. Letters indicate highly significant differences between OTU richness averages according to Tukey HSD post hoc test ($p<0,01$).
(B) factorial correspondence analysis to explain the projected variance. In black AM fungal communities for samples from the 1 plant species plots, from 2 plant species plots in red, from the 4 plant species plots in green and from 8 plant species plots in blue.
(C) linear regression of the fungal OTU richness in function of the plant species richness.

The results of our preliminary analysis seem to confirm our working hypothesis. The OTU richness decreases concomitantly with plant species richness (Figure 22A &C). Indeed, OTU richness in the plots featuring 1 and 2 plant species are the same and contain an average of 24 OTUs. The roots from plots containing 4 & 8 plant species contained a significantly higher number of OTUs (Figure 22A). This is further confirmed by the results of the correspondance analysis, which clearly shows that the number of OTUs is positively correlated with the plant species richness (Figure 22B).

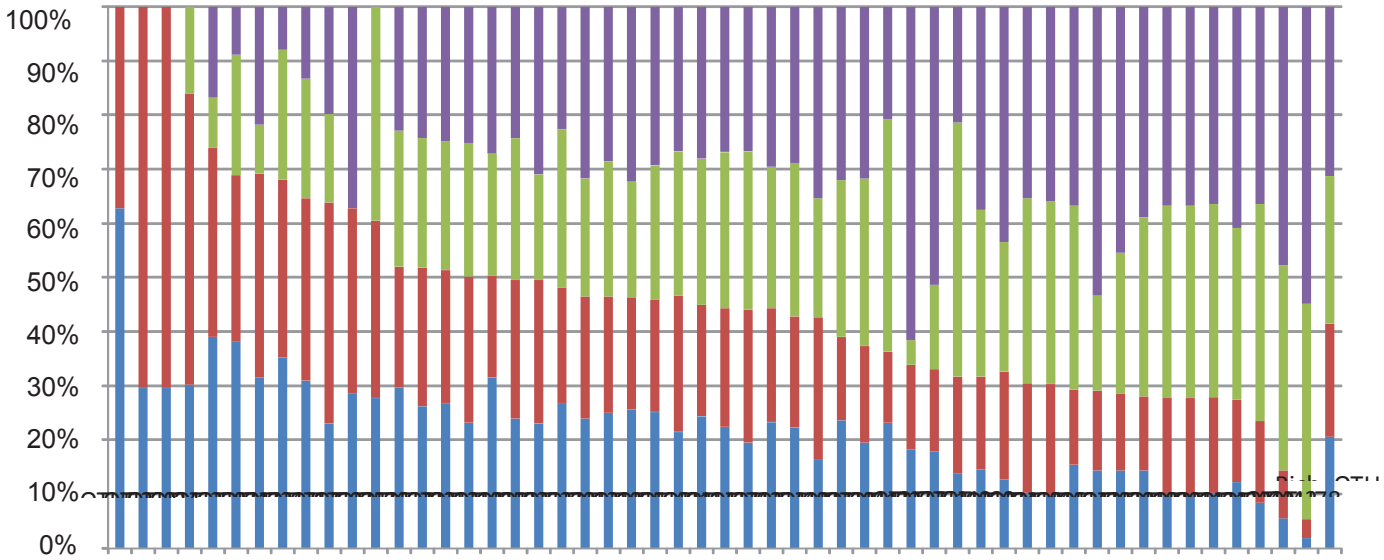


Figure 23:
Diagram showing the relative occurrence (presence/absence) of each of the 54 OTUs found in the study for the different modalities (1, 2, 4 and 8 plant species). In blue, red , green and purple are the OTU proportion observed in the 1, 2, 4 and 8 plant species modalities respectively.

The 54 different OTUs found in the study do not display the same abundance along the four plant species richness modalities (Figure 23) as some are rare and other are more common. The observed shift in the AM fungal community composition could be explained by changes in plant species richness. One third of the OTUs are found equally whatever the plant species richness (Figure 23, OTU 67 to OTU 54). Reciprocally, OTU 52, 69, and 24 were only detectable in the plots with 1 and 2 plant species, while OTU 78, 43, 40 were rare in the low plant diversity plots (i.e. with 1 & 2 plant species) and common in plots with 4 and 8 plant species.

OTU 52 was found in most of the low diversity plots (i.e. 75% of the sampled low plant diversity plots). Thus this OTU displayed a broad distribution only in low diversity plots, which may indicate a large host range. We can potentially explain the OTUs 69 and 24 presence only in a few plots with low plant diversity by suggesting that these AM fungi display a restricted host range. However this needs to be confirmed as it may be different at another period of the year. Another explanation is that these 3 OTUs (i.e. 52, 69 & 24) are only found in the 1 and 2 plant species plots because they are poorly competitive thus when the plant diversity increases, along with the emergence of a larger AM fungal diversity, these AM taxa fail to develop and spread.

OTUs 20, 43 and 78 belonging to the Acaulosporaceae and OTU 40 belonging to the Glomeraceae are frequent and mostly observed at a higher plant diversity levels (>4 plant species). Strikingly, the OTU 1 found equally in all modalities, is observed in 91% of the total samples and represents 64.3 % of the total number of sequences. If this frequency is not related to a preference of PCR amplification bias, the OTU should be considered as highly successful under these field conditions. This has to be confirmed by analysing interannual and seasonal changes in AM fungal communities.

A concordance index analysis (figure 24A) was performed on group OTUs displaying similar representations in the 4 different modalities. Figure 24A indicates 3 groups of OTUs that are all highly significantly different (Figure 24B, Kruskal Wallis: $p<0,001$) confirming that different AM fungal OTUs strategies exist across modalities. To draw the figure 24B, the average plant species richness of plots where each OTU was found, was performed for each group. OTUs within group 1 occurred at a lower average of plant species richness whereas group 3 occurred at the highest plant species richness average (i.e. group 1 average = 0.4; group 2 average = 1.6; group 3 average = 2.2)

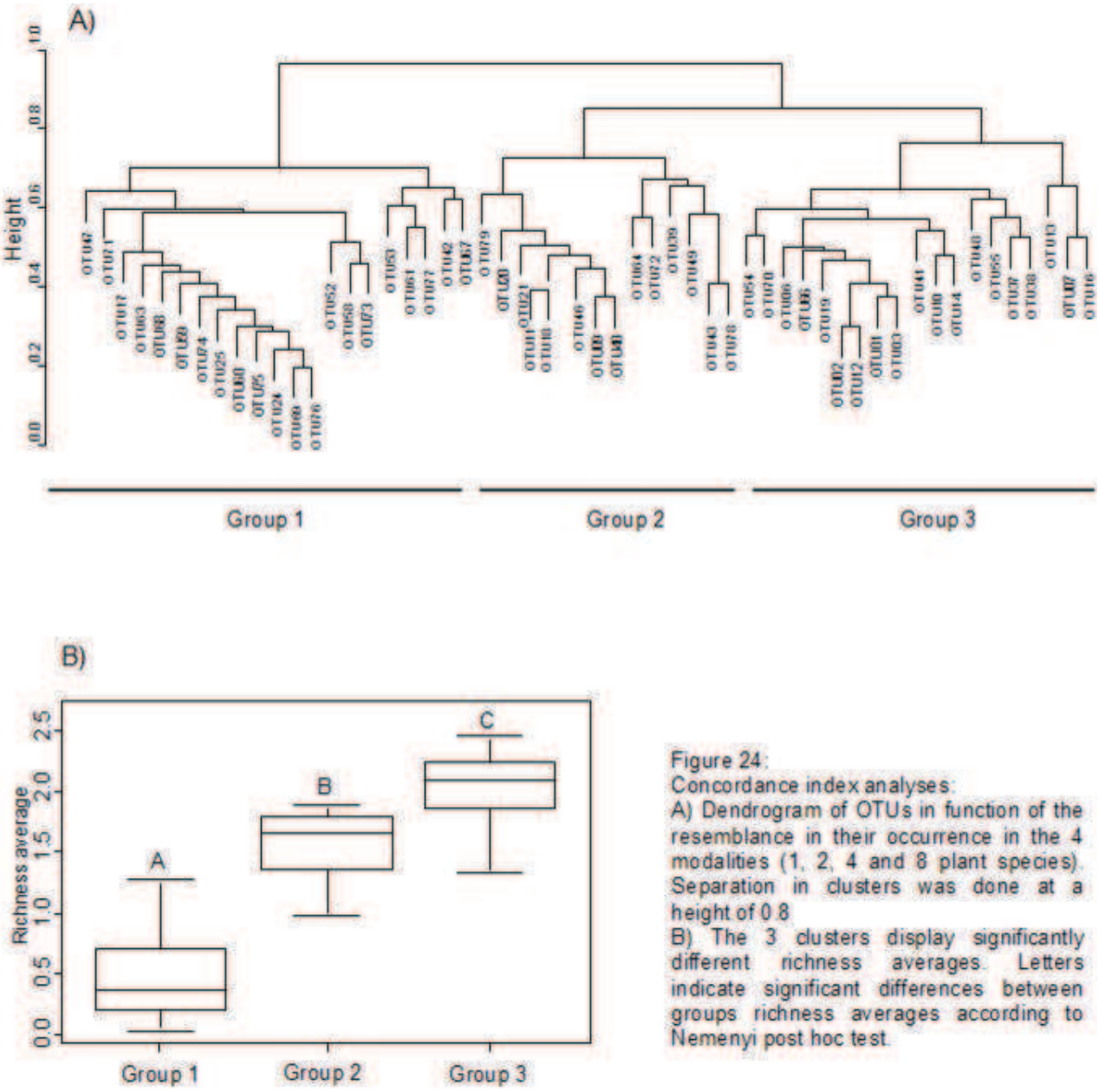


Figure 24:
Concordance index analyses:
A) Dendrogram of OTUs in function of the resemblance in their occurrence in the 4 modalities (1, 2, 4 and 8 plant species). Separation in clusters was done at a height of 0.8
B) The 3 clusters display significantly different richness averages. Letters indicate significant differences between groups richness averages according to Nemenyi post hoc test.

This additional analysis reinforced the above observations. Indeed OTUs 52, 69 and 24 were only found in the plots containing 1 and 2 plant species all belonging to group 1 (table3), whereas OTUs 78, 43, 40 and 20 mostly observed in the 4 and 8 plant species plots belong to the group 2 (table 3).

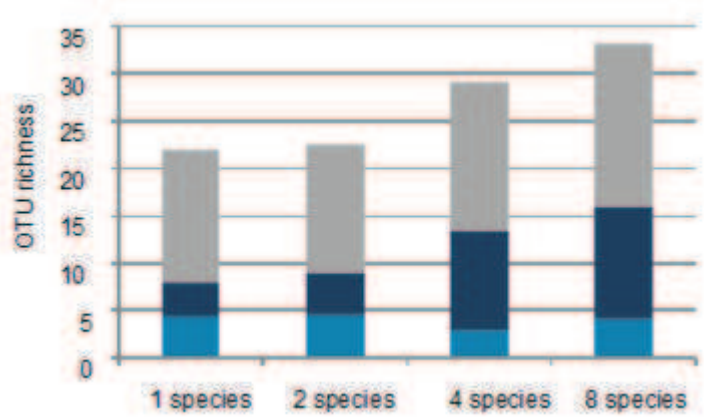


Figure 25:
Occurrence of the 3 clusters: group1, group2 and group3, depending on the plant species richness (1, 2, 4 or 8 plant species). Groups 1, 2 and 3 are respectively shown in light blue, dark blue and grey.

The proportion of each group within the 4 modalities (figure 25) suggests that group 1 contains the 'rare' OTUs. They are observed in 0.08 to 48.7 % of the samples and represents 0.001 to 0.1% of the total number of sequences (table 3). Group 3 contains OTUs with an intermediate abundance as they are found in 28.8% to 57.7% of the samples and covered 0.02% to 1.7% of the total number of sequences (table 3). OTUs within this group are mainly found associated with higher plant species richness. These results raise the question of the possibility of differences in between OTUs ecological status with more AM fungal generalists in low plant diversity levels and more specialists in the higher levels of plant species richness plots. In low plant diversity levels even if less frequent specialist AM fungi could also be present resulting in a higher variance in AM fungal community composition (i.e. root samples from low plant diversity modalities display a lower OTU diversity than samples from the 4 & 8 plant species modalities but the AM fungal composition from one sample is different from the composition of another sample) (figure 20B). The second possible interpretation of this higher variance is a variability among AM fungal community diversity existing among host-plants with plants being colonised either by a low or a high diversity of AM fungi: within the low plant diversity modalities, some plants are colonized by a high richness of AM fungi while others are colonized by a low richness of AM fungi. When plant species richness increases, more plant functional groups are likely to coexist, leading to a higher number of AM fungal habitats. Niche complementarity along with a higher functional diversity can explain a higher AM fungal richness and the possibility of the occurrence of AM fungal 'specialists' would explain the observed lower variance (figure 20B) in AM fungal communities.

OTU	% of samples containing it	% of seq tot	Family	Group
OTU 17	19,2	0,06	Diversisporales archaeospora	1
OTU 24	3,4	0,007	Glomerales glomeraceae 1	1
OTU 25	8,3	0,008	Diversisporales archaeospora	1
OTU 42	41	0,08	Glomerales glomeraceae 2	1
OTU 47	33,9	0,1	Glomerales glomeraceae 1	1
OTU 52	28,8		Glomerales glomeraceae 2	1
OTU 53	48,7	0,09	Glomerales glomeraceae 2	1
OTU 58	15,4	0,04	Glomerales glomeraceae 2	1
OTU 59	12,1	0,02	Glomerales glomeraceae 1	1
OTU 60	5,1	0,005	Glomerales glomeraceae 2	1
OTU 61	33,3	0,07	Glomerales glomeraceae 1	1
OTU 63	14,7	0,03	Diversisporales archaeospora 1	1
OTU 67	41	0,1	Glomerales glomeraceae 1	1
OTU 68	1,9	0,01	Diversisporales acaulosporaceae	1
OTU 69	0,08	0,001	Glomerales glomeraceae 2	1
OTU 71	30,1	0,09	Glomerales glomeraceae 2	1
OTU 73	22,4	0,03	Glomerales glomeraceae 2	1
OTU 74	8,3	0,006	Diversisporales acaulosporaceae	1
OTU 75	5,7	0,005	Glomerales glomeraceae 2	1
OTU 76	1,9	0,009	Glomerales glomeraceae 1	1
OTU 77	27,5	0,03	Glomerales glomeraceae 2	1
OTU 9	51,3	0,9	Glomerales glomeraceae 2	2
OTU 11	57,7	0,2	Glomerales glomeraceae 1	2
OTU 18	53,2	0,6	Glomerales glomeraceae 1	2
OTU 20	49,3	1,7	Diversisporales acaulosporaceae	2
OTU 21	46,8	0,7	Glomerales glomeraceae 1	2
OTU 39	42,9	0,35	Diversisporales diversisporaceae	2
OTU 40	51,3	0,4	Glomerales glomeraceae 2	2
OTU 43	34	0,1	Diversisporales acaulosporaceae	2
OTU 46	53,2	0,5	Diversisporales gigasporaceae	2
OTU 49	30,7	0,4	Glomerales glomeraceae 1	2
OTU 64	57,7	0,1	Glomerales glomeraceae 1	2
OTU 72	43,6	0,07	Glomerales glomeraceae 2	2
OTU 78	28,8	0,02	Diversisporales acaulosporaceae	2
OTU 79	30,7		Glomerales glomeraceae 2	2
OTU 1	91	64,3	Glomerales glomeraceae 2	3
OTU 2	94,8	4,3	Glomerales glomeraceae 2	3
OTU 3	93,5	7	Glomerales glomeraceae 2	3
OTU 6	80,2	1,6	Glomerales glomeraceae 2	3
OTU 7	45,5	0,3	Paraglomerales paraglomus	3
OTU 10	80,7	1,7	Glomerales glomeraceae 2	3
OTU 12	92,3	4	Glomerales glomeraceae 1	3
OTU 13	67,3	0,5	Glomerales glomeraceae 2	3
OTU 14	78,2	1,3	Glomerales glomeraceae 2	3

OTU	% of samples containing it	% of seq tot	Family	Group
OTU 16	60,2	0,7	Paraglomerales paraglomus	3
OTU 19	84,6	0,2	Diversisporales scutellosporaceae	3
OTU 37	73,7	0,4	Glomerales glomeraceae 1	3
OTU 38	71,8	0,2	Glomerales glomeraceae 2	3
OTU 41	71,8	0,4	Glomerales glomeraceae 2	3
OTU 48	67,3	0,2	Diversisporales scutellosporaceae	3
OTU 54	73,1	0,4	Glomerales glomeraceae 2	3
OTU 55	66	0,3	Glomerales glomeraceae 2	3
OTU 66	82	0,5	Glomerales glomeraceae 2	3
OTU 70	71,8	0,3	Glomerales glomeraceae 1	3

Table 3:
OTUs list and % of samples containing these OTUs, % of the total number of sequences they represent and taxonomical family within the Glomeromycota phylum.

It is important to again stress the preliminary nature of these results. In order to confirm these findings, the work will need to be refined by (i) analysing the inter-annual changes (ii) the seasonal changes (iii) analysing in more detail the link between host-plant species and the AM fungal community composition (iv) analysing the whole fungal community associated to roots, not solely AM fungi (v) from a technical point of view making the number of produced sequence per sample analysed more homogeneous to allow the use of a matrix of relative frequencies (vi) including the 16 plant species modality within the analysis shown herein (sequencing under process). Despite the limitations of this current work, two ideas emerge: (i) in agreement with our working hypothesis, we found evidence that a decline in plant diversity induces a decline in AM fungal species diversity and (ii) a higher plant diversity will induce a convergence in the qualitative composition of the AM fungal communities.

Modeling of the root associated community by means of computing correlation networks will be performed to predict the key component(s) within this community. This should provide important information about community functioning, including synergies and competition among fungal communities, which are currently poorly documented.

Supplementary material:

324	323		286	285		248	247			210	209		172	171		134	133			096	095		058	057		020	019
325	322		287	284		249	246			211	208		173	170		135	132			097	094		059	056		021	018
326	321		288	283		250	245			212	207		174	169		136	131			098	093		060	055		022	017
327	320		289	282		251	244			213	206		175	168		137	130			099	092		061	054		023	016
328	319		290	281		252	243			214	205		176	167		138	129			100	091		062	053		024	015
329	318		291	280		253	242			215	204		177	166		139	128			101	090		063	052		025	014
330	317		292	279		254	241			216	203		178	165		140	127			102	089		064	051		026	013
331	316		293	278		255	240			217	202		179	164		141	126			103	088		065	050		027	012
332	315		294	277		256	239			218	201		180	163		142	125			104	087		066	049		028	011
333	314		295	276		257	238			219	200		181	162		143	124			105	086		067	048		029	010
334	313		296	275		258	237			220	199		182	161		144	123			106	085		068	047		030	009
335	312		297	274		259	236			221	198		183	160		145	122			107	084		069	046		031	008
336	311		298	273		260	235			222	197		184	159		146	121			108	083		070	045		032	007
337	310		299	272		261	234			223	196		185	158		147	120			109	082		071	044		033	006
338	309		300	271		262	233			224	195		186	157		148	119			110	081		072	043		034	005
339	308		301	270		263	232			225	194		187	156		149	118			111	080		073	042		035	004
340	307		302	269		264	231			226	193		188	155		150	117			112	079		074	041		036	003
341	306		303	268		265	230			227	192		189	154		151	116			113	078		075	040		037	002
342	305		304	267		266	229			228	191		190	153		152	115			114	077		076	039		038	001

Figure S12: Plot numbering of the e120 experiment in Cedar Creek LTER.

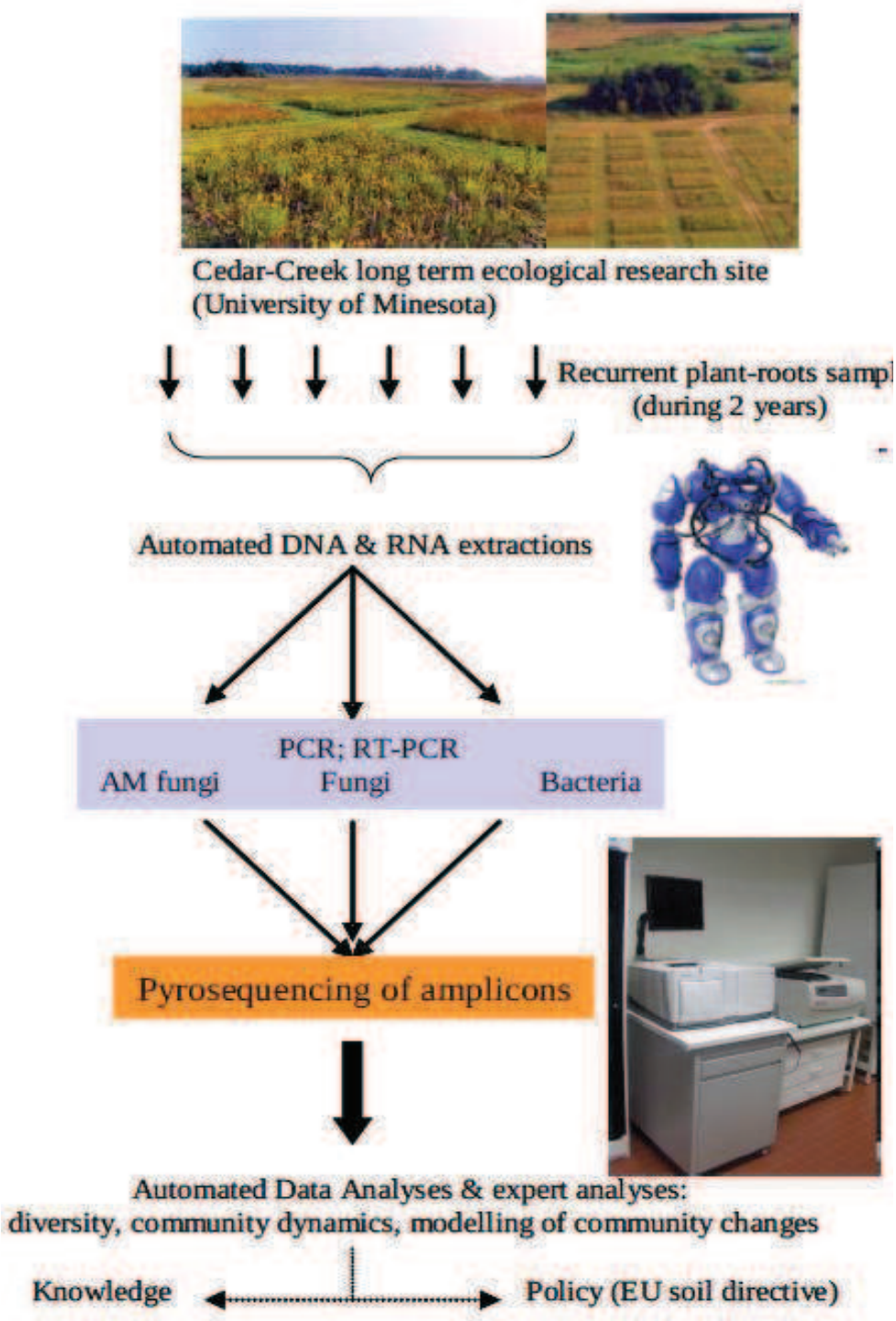


Figure S13:

Strategy to analyse the diversity of the root associated microorganisms. In this chapter are only presented the results of the PCR targeting AM fungi.

Chapter V

Global Sustainable agriculture : new possible trajectories from mutualistic symbiosis

Marie Duhamel[1,2], Philippe Vandenkoornhuyse[1].

[1]Université de Rennes I, CNRS UMR6553 EcoBio, Campus Beaulieu, F-35042 Rennes, France.

[2]Institute of Ecological Science, Vrije Universiteit, 1081 HV Amsterdam, Netherlands

Submitted to Trends in Plant Science (2013)

Summary

Food demand will increase concomitantly with human population but reports indicate that agricultural productivity will decrease as a result of global warming and soil degradation. To feed the world, food production therefore needs to be high enough and, at the same time, minimize damage to the environment. This equation cannot be solved with current strategies. Based on recent findings on the control of the AM symbiosis, new trajectories in soil management practices for agriculture and plant breeding which take into account the below-ground compartment and evolution of mutualistic strategy, are proposed here. In this context, we argue that plant breeders have the opportunity to make use of native Arbuscular Mycorrhizal symbiosis in an innovative ecologically intensive agriculture.

Introduction

Feeding the world and securing access to food are both major social and scientific issues. In recent years, the rapidly increasing demand for food (i.e., for human populations and livestock) along with biofuels has led to food price volatility (Battisti & Naylor, 2009). Recent work suggests that food crises are even more exacerbated by global warming: agricultural productivity has declined world-wide as a consequence of the hottest summers experienced in the recent past, and according to different global warming scenarios […] *the hottest seasons on record will represent the future norm in many locations* [...] (Battisti & Naylor, 2009). Human population has increased and will continue to increase to a peak, expected before the end of the century, with 10 billion people before 2100 (Lutz *et al.*, 2001). Contrary to common assumption, non-linearities between population expansion and environmental degradation are likely to increase disproportionately and rapidly (Harte, 2007). Human population expansion will be coupled with an increased demand for space, water and food. These demands will therefore be accompanied by urban and cropland expansion, and more than 10^9 hectares of natural ecosystems are likely to be lost by 2050 (Tilman *et al.,* 2001). This represents collateral damage for the environment because cropland expansion can only be achieved by replacing non agricultural, mainly forested areas. According to recent studies, agricultural production will have to expand by about 100% during the 21st century to satisfy forecast world demands (e.g., Cirera & Masset, 2010). At the same time, agriculture is a major threat to the environment, leading to a decline in biodiversity and related ecosystem services, including degradation of soil and water quality (e.g., Foley *et al.*, 2005).

A fundamental issue for agriculture during this century is to confront two contradictory goals, (i) the need to produce enough food to minimize human malnutrition and support world population expansion and (ii) the need to limit collateral damages to the environment, which can in turn negatively impact agriculture. Based on recent findings about strategies in plant mutualisms and plant selection, our aim is to propose new ideas and suggest guidelines for sustainable agricultural development.

Intensive vs. extensive agriculture ?

To achieve a sustainable agriculture, the need is to increase the productivity while limiting the inputs in fertilizers and biocides and the damages to the environment. In this

context, both intensive agriculture and extensive agriculture should be considered. The aim in intensive agriculture is to maximize productivity per unit of surface while in extensive agriculture, lower productivity yields are accepted as a counterpart to less potential ecosystem damage. The main advantage of extensive agriculture is that no or few inputs are required. However this is often countered by a need for larger soil area to obtain comparable production. It has been shown that agricultural intensification with high yield production eventually increases greenhouse gas emissions per unit surface. However much higher emissions can be expected if the same production is obtained by expanding low-yield farming (Burney *et al.,* 2010; Balmford *et al.,* 2012). Similarly, the need to increase agricultural productivity to limit adverse effects on the environment has also been underlined by modeling land use/land cover changes (Nelson *et al.*, 2010) and by projecting possible improvements of productivity in existing agricultural areas (Foley *et al.*, 2011). One key element which has emerged is the necessity for agricultural intensification to preserve biodiversity and the related ecosystem services.

Crop selection from traits?

Since the beginning of agriculture, crops have been selected for different traits, including plant productivity. The main current approach to modern plant breeding is to maximize the fitness of individual plants. However other contrasting breeding strategies have been suggested. One of the most exciting of these new solutions would be to base plant breeding on group selection rather than on individual plant fitness (Weiner *et al.*, 2010). This would imply a completely new approach to selection criteria involving the maximization of population performance and not of the individual peformance, this can produce higher yields. For example, selecting for cooperative shading, which would allow a passive control of weeds, seems promising to improve yield and sustainability (Weiner *et al.*, 2010).

In these two approaches, however, the belowground compartment is not considered, which is arguably a mistake. Plants are deeply dependent on mutualist microorganisms for their growth, and these can be damaged by conventional agricultural practices and current plant breeding strategies. For example, conventional agriculture causes a decrease and a uniformisation of AM fungal taxa (Verbruggen *et al.*, 2010), Another consequence can be the spread of unusual 'behavior' of arbuscular mycorrhiza in monocultures. They act like parasites which causes a decrease in crop yield (for a review

see Bennet *et al.,* 2011).

Arbuscular mycorhiza and consequences of agriculture

The arbuscular mycorrhiza relationship evolved roughly 400 million years (Redecker *et al.,* 2000). This symbiosis is widespread with approximately 80% of land plants colonized by AM fungi (Smith & Read, 2008), across a huge diversity of ecosystems. In this symbiosis, plants provide carbohydrates to the arbuscular mycorrhizal fungi in exchange for minerals, drought resistance and protection against pathogens (Smith *et al.*, 2010). The fungus in this mutualistic relationship is an obligate biotroph, its transmission is horizontal as symbionts are taken up from the environment and there is no genetic uniformity between fungal symbionts as the nuclei coexisting in the AM symbionts are genetically dissimilar. Several different fungal symbionts colonize the same plant roots.

The arbuscular mycorrhizal symbiosis is responsible for massive global nutrient transfer. It is a mutualism 'that helps feed the world' (Marx, 2004). Arbuscular mycorrhizal fungi, because of their functions, can be considered as key microorganisms for soil productivity.

Intensive agricultural management (i.e., conventional agriculture in Europe and North America) has exerted a high selection pressure on microorganisms through profound modification of their habitats and niches, notably brought about by tillage, the high increase of mineral nutrients, and low plant diversity (i.e., crops). Tillage, ploughing and ripping, for example, represent an intense form of soil disruption. In natural habitats, AM mutualism is not subjected to perturbations of this intensity. Such disruption leads to degradation of the hyphal network, ecological functions, and AM fungal diversity (for review, see Verbruggen & Kiers, 2010). Soil nutrient availability is a strong driving influence for producing an evolved geographic structure in AM mutualism (i.e., a coevolutionary selection mosaïc) (Johnson *et al.*, 2010). As a result, soil fertilization in agricultural ecosystems has had a negative impact on AM fungal functions (e.g., Johnson, 1993) and diversity (Egerton-Warburton *et al.*, 2007). Recently, Sheng *et al.* (2012) have shown that tillage and phosphorus fertilization have different and additive effects on AM fungi as tillage decreases arbuscular development in roots while phosphorus supply reduces the total AM fungal colonization. Thus confounding factors, related to conventional agricultural trajectories, act synergistically against mycorrhizal symbiosis.

Mutualistic strategy and agriculture

From a theoretical point of view, mutualisms (cooperative interactions among different species) can exhibit instability: individuals potentially benefit from defecting from cooperation if cooperation is costly. Organisms will increase their own fitness, even if this comes to a cost of others. This means that less-cooperative strains (i.e. 'cheaters') Kiers *et al,* (2011) have demonstrated the capacity of plants to sanction symbiont of low quality providing them less carbon . Thus the gain in fitness for the cheater is reduced by this plant trait. This in itself can explain the stability of this symbiosis. A similar sanction of carbon allocation has been observed in the case of nitrogen-fixing nodules in leguminous plants to control *Rhizobium* cheaters (Kiers *et al.,* 2006).The most cooperative AM fungal symbionts transfer more phosphorus to the roots when they receive more carbon (Kiers *et al.*, 2011). Such mutualism is therefore bilaterally controlled because both partners can enforce the cooperation and any possible enslavement strategy is also limited. This fairly explains the stability of arbuscular mycorrhizal symbiosis. In addition, the main advantage for the plant to not enslave its symbionts is this access to numerous potential functions harbored by the reservoir of soil AM fungi into which the plant can tap depending on its nutritional requirements. For the fungi, the main outcome of not being enslaved is the maintaining of a a high level of diversity. This symbiosis is one reason for the success of plants in terrestrial ecosystems.

Less cooperative AM fungi do exist in nature. We can expect them to become more abundant as the diversity of AM fungi decreases because the symbiotic options offered to the plants are more limited. Kiers *et al.* (2011) found that AM fungi cheaters can develop 'dealer' strategy by keeping phosphorus in polyphosphate chains and delivering it at an expensive cost for the host plant. The plant's capacity to sanction cheaters is a tremendously important trait to maintain, given the fact that most mineral nutrients (~70% of the phosphorus for example) are delivered to plants by AM fungi (Smith *et al.*, 2003).

Ecosystem productivity has been shown to be responsive to AM symbiosis diversity (van der Heijden *et al,* 1998; 2007). Host plants can be colonized by a variety of AM fungi (i.e., no host-specificity). However, recent findings suggest that plants can selectively allocate more resources to those fungal partners that ensure access to particular functions related to their needs (Kiers *et al.,* 2011). This 'selective rewarding' is likely to lead to the exclusion of certain colonizers and culminate in an observed 'host-plant preference'

(Vandenkoornhuyse *et al.*, 2002; 2003).

This leads to the idea that a plant can filter soil AM fungi depending on its requirements, the season and location. Conventional field-based agriculture makes use of very limited crop plant diversity, fungicides, soil tillage and fertilizer. The pressure exerted by agricultural practices leads to a reduction in AM fungal diversity compared to more natural ecosystems (e.g., Helgason e*t al*., 1998; Verbruggen *et al.,* 2010). Breeders generally select crop cultivars from rich soils which have been under conventional agriculture for many years. Agricultural soils have been enriched with fertilizers for decades and the ecological function of AM fungi as a provider of plant phosphorus is less important in these enriched soils. This can, in turn, relax selection for traits that allow plants to best evaluate their symbiotic partners. For example, it has been shown that older soya bean varieties are better able to control *Rhizobia* cheaters than modern soya bean cultivars (Kiers *et al.*, 2007).

The same trend has occurred in the plant-mycorrhizal mutualism. There is work suggesting that AM fungal cheaters increase in agricultural soils. A loss of mycorrhizal responsiveness due to modern plant breeding was shown in wheat and maize (An *et al.*, 2010; Zhu *et al.,* 2001) In breadfruit, the selection of cultivars favoring above-ground traits can lead to a defection of the AM mutualism (Xing *et al.,* 2012). Because AM fungi constitute a fundamental component of soil fertility, solutions for a more ecologically intensive agriculture should focus on this research question. Plant breeders could imagine new selection trajectories where the sanction trait is considered as a major selection target. In this way the possibilities offered by AM functional efficiency could be restored and agricultural practices modified by reducing soil inputs and tillage.

The alternative hypothesis is that plant breeders have selected cultivars that are very efficient for mineral foraging through soil AM fungal mutualists. This apparently optimistic hypothesis is worse than that of a loss of the sanction trait in crops, because of the lack of long term sustainability. Indeed, if there is a loss of AM fungal diversity and quality due to the low plant diversity in agroecosystem, plant would have less chance to meet their need. Furthermore, one important component of soil fertilizer, phosphorus, is known to rely on high quality rock phosphate, which is a finite resource. More than 85% of the global phosphate resources are dominated by only 3 countries which is far fewer than

the number of countries controlling the world's oil reserves (e.g., Elser & Benett, 2011). Phosphorus supply is thus of strategic importance for many countries, and [...] *many food producers are in danger of becoming completely dependent on this trade* [...] (Elser & Benett, 2011). Major agricultural regions such as India, America, and Europe are already dependent on P imports. Phosphate market prices can soar, as shown by the 700% increase in 2008 (Elser & Benett, 2011), especially as phosphate mining production is predicted to attain a peak in 2030 (Cordell *et al.*, 2009).

Other plant mutualisms, in addition to arbuscular mycorrhiza, should potentially have a synergistic impact on plant productivity and plant resistance against stresses. For example, infection of barley with an endophytic fungus, *Piriformosa indica*, increases resistance to stresses including salinity and systemic resistance of the crop to root and leaf pathogens, and a concomitant increase in yield production (Waller *et al.,* 2005). Native plants in coastal environments and geothermal habitats require fungal endophytes in order to grow (Rodriguez *et al.*, 2008). Thus a passive adaptation of the plant is observed, with the endophytic fungus providing a selective advantage to the colonized plant. Infection of the tomato plant with these endophytes, for example, confers salt or heat resistance (Rodriguez *et al.*, 2008). It can thus be argued that solutions, which support a more productive and sustainable agriculture and involve the use of endophytic microorganisms, do exist but have as yet been little explored.

Future of agricultural trajectories

Forests represent important carbon stocks which, when converted into agrosystems, have a huge impact on CO2 emission to the atmosphere (e.g. Aldhous, 2004) as well as a collateral effect on biodiversity (e.g. Balmford *et al.,* 2012). In the context of global changes, it seems fundamental to limit agricultural expansion (Foley, 2011). The key point seems to be to improve crop yields within existing agrosystems. However, conventional agricultural practices and plant breeding strategies have arguably entered a 'cul-de-sac' because they are [...] *unlikely to improve attributes already favored by millions of years of natural selection* [...] (Weiner *et al.,* 2010) while under-explored natural keys to crop yield improvement , such as AM fungi exist but are ignored and maltreated.

To maintain or restore this essential component of soil fertility, conventional

agricultural practices need to be modified. The following are suggested guidelines to improve the sustainability of human land use and crop productivity:

(1) Because AM diversity is positively correlated with plant diversity (van der Heijden *et al.,* 1998), agriculture will need to make use of greater plant diversity. (2) Tillage, if employed, will need to be restricted to maintain hyphal networks and functional efficiency and also to preserve soil aggregates and limit water losses (Souza-Andrade *et al.*, 2003). (3) Plant breeders should select plants in poor soils, taking into account the 2 previous aspects, the aim being to maximize the efficiency of AM fungi symbiosis (i.e., plants able to take full advantage of the AM fungi available in soils). These new selected plants might also be able to restore effective AM fungi in the field (4) Additional mutualist microorganisms such as endophitic fungi should also be considered as important targets to improve plant resistance and productivity.

This should facilitate a promotion of AM fungal mutualism and, at the same time, reduce the use of fertilizers, biocides and water. These guidelines have the potential to enhance crop yields and reduce the problems associated with conventional agriculture in both developed and developing countries.

Conclusion

The Green Revolution started about 50 years ago and has allowed food shortages to be limited. Given the stocks of resources and human population growth, this Green Revolution can continue for only a few more decades. The counterpart of this Green Revolution is a high cost to the environment and global environmental changes (e.g., Tilman *et al.*, 2001). If nothing is done to counteract these changes, thresholds will be exceeded, with dramatic consequences (Harte, 2007) and indeed the impossibility for natural ecosystems to regenerate. A more sustainable agriculture has to emerge to guarantee food supply over the next 50 years. One way of achieving a more ecologically intensive agriculture would be to consider and protect the ecological functions displayed by AM fungi. This will not only improve natural plant mineral nutrition but also water supply and other ecological functions that have already been clearly documented (e.g. Smith & Read, 2008).

Acknowledgements

*The work is supported by a grant from 'l'Agence Nationale de la Recherche' (*ANR-10-STRA-0002). We acknowledge ET Kiers and D Warwick for comments and suggestions of modifications on previous versions of the manuscript.

General Discussion and Perspectives

The aim of this thesis was to address key aspects governing the arbuscular mycorrhizal symbiosis. To achieve this goal, I executed a series of analyses and experiments ranging from the individual host-plant level to the plant community level. We examined discrimination processes, asking if plant and fungal partners were able to detect cooperation, i.e. each other's level of nutrient provision. We then studied the strategies that a host plant may deploy to protect its cooperating AM fungal symbionts. We developed and published for the public a sequence database to ensure proper analysis of fungal identity. We also used emerging molecular methods to investigate the effects of variation in host plant species richness and diversity on the AM fungal communities. Below, I give a summary of these chapters and results.

I. Exploring the bilateral control of AM symbiosis through preferential C allocation and P hoarding strategies

The maintenance of cooperation in the mycorrhizal partnership poses a problem for evolutionary theory. The problem is particularly puzzling because both plant and fungi interact with multiple partners simultaneously: a single host plant is colonized by multiple fungal species and fungal 'individuals' interact with multiple host plants. This complex series of many-to-many interactions means that neither partner can be 'enslaved'. It also means that selfish individuals can exploit the relationship, reaping benefits while paying no costs, so why cooperate at all? In chapter I, we used stable isotope probing techniques and tracking of radioactive elements in *in vitro* root systems to demonstrate that plant and fungal partners are able to detect variation in nutrient provisioning by the other, and adjust their own strategy accordingly. We argued that the partnership functions like an economic market: partners compete by trading resources, and those offering the best rate of exchange are rewarded. We also found that some species of AM fungi stock P in host-inaccessible polyphosphate chains and adopt a hoarding strategy. Whether these same processes operate under natural conditions are unknown.

1. Functioning of AM symbiosis in a more complex experimental design

Our experiments utilized simplified conditions with three AM fungal species and one *Medicago truncatula* host grown under laboratory conditions. As AM fungal functioning can be very context dependent, lab and field experiments need to be combined to look at increasing levels of complexity, and realism. As a natural environment is generally composed of a broad diversity of AM fungi displaying different colonization strategies, the first step should be to enlarge the number of AM fungi tested. This could be achieved by performing additional stable isotope probing experiments. For example, other AM fungi from different families displaying different root colonization strategies, like Gigasporaceae or Acaulosporaceae, could be tested. Gigasporaceae fungi display high soil colonization but low roots colonization whereas Glomaceae exhibit the opposite characteristics, while Acaulosporaceae exhibit low colonization rates in both roots and soil (Hart & Reader, 2002). Molecular markers need to be developed for strains from these fungal groups and tested to be able to track these species in multi-species communities.

2. Breeding and the ability of plants to discriminate among fungal partners

An outstanding question is how crop breeding has changed the ability of hosts to discriminate among their fungal partners. Future experiments should use a SIP-RNA approach to look at host carbon allocation patterns across crop cultivars (from wild genotypes to high-bred, recently released cultivars). Plants would be grown without AM fungi, colonized by high-quality strains, low-quality strains and in a mixture containing both types of symbionts. I would hypothesize that if the plant has lost its ability to discriminate, the biomass of a host plant grown in the fungal mixture will be similar to that of plants grown in the presence of only low-quality fungi. Conversely, if the plant biomass is similar to that of plants grown in the presence of high-quality strains, this would suggest that the capacity to sanction non-cooperative strains is conserved. This could be confirmed by studying carbon allocation patterns in the mixed treatment.

After studying the effects of the host, we could then investigate the effects of the fungus. Plants could be inoculated with fungal spores harvested from plots under conventional agricultural monocultures (i.e. exposed to fertilizers, biocides and tillage), to test whether conventional agricultural practices select for less mutualistic fungal partners

(chapter V). For a large range of agricultural plants, the functional effects of AM fungal colonization are still unknown. It is important to determine which plants profit from AM fungi, and to determine their level of dependency to be able to enhance agriculture management and soil restoration.

3. Plasticity of the AM symbiosis

It is well known that for the same AM fungal strain, the impact of the symbiosis on plant fitness will differ depending on the host plant species. Reinhart *et al.* (2012) tried to predict plant responses to AM fungal colonization using a plant phylogeny approach. However, they found it impossible to predict functional consequences and interactions using this approach. Phylogenetic proximity has been shown to be a poor predictor of plant responses to AM fungi. A major question is whether AM fungi are more or less adapted to particular plants? For example, it has been shown that plants inoculated with AM fungi from their native soil exhibit more arbuscules in their root cells, and that these native AM symbionts perform best in their endemic soil (Johnson *et al.,* 2009). Studying adaptation in AM fungi is difficult because spores and hyphae contain hundreds of nuclei, some of which can vary genetically. Nuclear sorting could result in high functional plasticity (Ehinger *et al.,* 2009). AM fungi are also able to form anastomoses through which genetic material can be exchanged. This makes it difficult to predict the consequences of particular host-fungal combinations.

4. AM symbiosis costs and related hypotheses

When studying mutualisms, the cost:benefit ratios of partnerships need to be well understood. In the AM symbiosis, one approach is to study the ratio between the phosphorus benefits provided by the fungi and the cost in carbohydrates allocated to the fungus, even though this does not represent all the diverse benefits AM fungi can provide. AM fungal associations are costly to the plant at the early stage of its growth. The carbon drain to the roots, due to their rapid colonization by AM fungi, can be massive enough that it reduces plant growth (Olsonn *et al.,* 2010). Carbon allocation to the fungal partner also represents a high cost for the plant when the amount of light is low (Fitter *et al.,* 2006). However, if the carbon allocated by the host plant to its symbiont comes from resources in excess, this carbon flux cannot be considered as a cost. For example, the carbon cost

might be balanced by the increased photosynthetic rate, which is stimulated by the C sink strength of the symbiosis (Kashuk *et al.,* 2009). However this phenomenon which offsets the cost of AM symbiosis is not generalizable (Black *et al.,* 2000). It depends on the growth stage of the plant and on the colonization stage of the fungus. Furthermore, the C cost of the symbiosis can be masked by the beneficial nutritional effects provided by AM fungi (Kashuk *et al.,* 2009). The question of luxury resources exploitation (e.g. Kiers & van der Heijden, 2006) should be further investigated. However, the use of excess plant carbon is likely only transitory and dependent on plant growth stage and nutrient availability.

II. Physiological and molecular bases of the sanction trait by plants

While we found strong evidence for the capacity of *Medicago truncatula* to discriminate among AM fungal strains (Chapter I), the molecular and physiological bases of this phenomenon is still unknown. Future experiments are needed to reveal the molecular mechanisms behind patterns of preferential carbon allocation. One way to address the question would be to use a sequenced plant like *Medicago truncatula*. It should then be possible to perform a comparative transcriptomic profiling (i.e. RNA seq and microarrays) on microdissected root parts. The easiest way to apply this molecular strategy would be to grow plants colonized by both high- and low-quality fungal strains. By choosing target genes involved in mineral transfer, carbon transfer, transportation, etc, it should be possible to analyse their differential expression/transcription (underexpressed *vs* overexpressed) under different fungal and control treatments. These gene expressions should be monitored over time: before colonization by AM fungi, during the establishment of symbiosis and after colonization. However, the feasibility of this approach remains to be proven because of (i) the instabilities of RNAs, difficulties in conserving material, and the small amount of RNAs obtained after the microdissections and (ii) the possibility that 'contaminant' plant RNAs from cells close to the arbuscules masks the molecular signal.

Transcriptomic analyses should also be performed to study the fungal side of AM symbiosis control, to see if similar mechanisms are present in other kind of symbionts like endophytic fungi, rhizobia etc. The hope is that a reference Glomales genome will soon be available. However, the genetics of AM fungi present a real challenge. Scientists are now achieving a better understanding of AM symbiosis by using 'omic' tools (Salvioly & Bonfante, 2013). Molecular biology has been used to detect the signals involved in

symbiosis establishment and in nutrient exchanges. We currently have transcriptomic datasets on the reprogramming of plant genes activity induced by symbiosis establishment (for review see Sanders & Croll, 2010). However these transcriptomic studies have been conducted, almost exclusively, on plant roots or leaf cells in response to symbiont colonization but rarely on the changes of gene expression in AM fungi in response to interaction with roots. This could be approached by using orthologous genes from other fungi also infecting plants. Up to now, only two mitochondrial genomes, several unrelated nuclear genes of AM fungi and the transcriptome of *G. intraradices* have been published (Franz & Hijri, 2009; Formey *et al*., 2012).

III. Host plant involvement in AM symbiont defense

In the second experiment (Chapter II), the functioning of the AM fungal symbiosis was examined in a multi-species context. In natural ecosystems, plants and AM fungi are embedded in a species network, and their relationships are affected by these other species. To understand the functioning of the AM fungal symbiosis, we increased the complexity of the system by adding fungivores. We hypothesized that the plant could aid in the defense of its symbiont, and thereby indirectly promote its own fitness. We found that a well known plant secondary metabolite, catalpol – a known fungivore repellent – was found in the mycorrhizal hyphae. However, its presence was only detectable when there were fungivores in the soil. We suggested there was a transfer from plant to fungus triggered in the presence of fungivores.

This transfer of secondary compounds was only tested in one plant species. Further tests are needed to expand these conclusions. This idea also needs to be tested using symbionts that vary in their quality. Although we tried to investigate whether the transfer of compounds differed depending on the identity of the fungal species, the inoculation with the low-quality fungal species failed. Our aim was to determine if the amount of secondary metabolite transfer is dependent on the quality of the fungal species: do plants allocate a lower amount of secondary metabolites to low-quality symbionts, thus allowing fungivores to graze the low-quality strains? A series of experiments is needed in which host plants are inoculated with a range of fungal symbionts differing in quality. We could then assess whether the transfer of secondary metabolites correlates with the quality of the fungal species. The range of secondary metabolites tested could also be enlarged to determine if plants can only transfer catalpol or if other compounds are involved in fungal protection.

Fungivore mortality is also a potentially interesting factor to study as a proxy to the intensity of the protection provided by the host plant. In addition, transcriptomic approaches could be utilized to more precisely understand the underlying mechanisms behind this transfer.

IV. Plant diversity versus fungal symbiont diversity

1. Molecular phylogeny and taxonomy

Studying AM fungal diversity based solely on morphological criteria is extremely difficult as they are not cultivable organisms, and because of phenotypic similarities/convergences among divergent organisms. Thus the use of molecular tools is the best solution to have document the range of fungal diversity. The development of new sequencing technologies and computational approaches has resulted in a significant step forward in the analysis of genetic and functional diversity. Diversity analyses based on metagenomic approaches and amplicon analyses involving high throughput sequencing are facilitating the assessment of both diversity and functions even for poorly known microorganisms. The use of next generation sequencing (NGS) has led to a tremendous increase in the amount of data obtained, and allowed for more in-depth analyses. In view of the size of the datasets, data analysis automation has become essential. An amplicon-sequence analysis pipeline (figure 18) has been developed using a web-based Galaxy instance for intensive computations (Bahin *et al.*, unpublished). With these new tools, known and unknown (micro)organisms within a community can be analysed after PCRs. In the study presented in Chapter IV, we used the primer set AM1-NS31 (Helgason *et al.*, 1999; Simon *et al.*, 1992) to analyse the AM fungal community and diversity. These community analyses now need to be improved by including other primer sets to limit the bias associated with preferential amplification.

In addition to the amplicon sequence pipeline developed during this thesis (chapter IV), a database Phymyco-DB (chapter III) was created to facilitate the sequence analyses. Phymyco-DB contains high quality fungal sequences of SSU rRNA and EF1α genes which have both been shown to be efficient in fungal identification. Phymyco-DB has been propagated within the Galaxy pipeline (Mahe *et al.*, 2012).

Working with Phymyco-DB underscored the poor quality, the lack of precision and the many errors that are currently found in public databases. All these factors lead to

erroneous taxonomies or at least to sequences not corresponding to the given taxonomy. Much work is still required to achieve reliable identification and assignment of Glomeromycota sequences. The traditional classifications based on morphological criteria can lead to numerous mistakes, and even the species concept currently utilized is questionable. Furthermore, the presence of spores does not necessarily indicate that the AM fungi are active. Numerous examples of misidentification of spores exist and significant problems exist with the naming and the molecular phylogenetic position of the fungi. For instance, an AM fungus classified as *Glomus versiforme* (culture line BEG47) in the 1980's is actually *Diversispora epigea*, but was only updated in 2011 by Schüßler *et al.* after molecular analysis. However, errors in the public sequence database (i.e. Genbank; Benson *et al.,* 2004) persist because of incorrect annotations and the propagation of mistakes. A good classification system associated with trustworthy RNA/DNA databases and analysis tools are the foundation for the description and understanding of phylogenic and functional trait diversity regulating plant/AM fungal associations, communities and productivity. A solid phylogeny is the basis of systematic analyses, establishment and understanding of the different hierarchical levels governing the taxonomic and functional diversity of organisms. In addition to the classic SSU rRNA gene usually targeted in microbial community analyses, other markers could be used, such as the large subunit of the rRNA gene (e.g. Clapp *et al.*, 2001) and the EF1α gene encoding elongation factor 1 (e.g. Helgason *et al.*, 2003). These two genes are more variable than the SSU rRNA gene. Thus, for organisms that emerged early, such as the Glomeromycota, these LSU rRNA and EF1α genes likely contain more homoplasic signals (i.e. inherited similarities). When the aim is to achieve reliable identification for closely related isolates, the mitochondrial LSU rRNA gene (=mtLSU rRNA; Thiéry, 2010, PhD Thesis, University of Basel) appears to be suitable (Kiers *et al.*, 2011). Conversely, the use of ITS as species-'barcoding' marker should be avoided for Glomeromycota because AM fungi are multinucleate and display different ITS copies (e.g. Sanders *et al.*, 1995; Boon *et al.*, 2010). By applying molecular taxonomies, which are much more reliable for determining the phylogenetic relationships between fungal taxa, unification of the Glomeromycota taxonomies should be possible.

2. Different levels of diversity to consider

In Chapter IV, the aim was to understand the functioning of the mycorrhizal symbiosis in a broader community context. The impact of plant discrimination processes

on fungal biodiversity is not well understood. This is because assessing the processes regulating the diversity of AM fungi in ecosystems is challenging. In Chapter IV, I investigated the link between plant and AM fungal symbiont diversity. Next generation sequencing and high throughput amplicon sequence analyses were applied to study the AM fungal diversity colonizing roots. This innovative molecular approach allowed us to handle a large number of samples and data. Roots were sampled from Cedar Creek Experimental Station in plots hosting a plant diversity gradient from 1 to 16 species. The goal was to observe the effect of a diversity of host on the AM fungal community structure. The project includes five sampling campaigns in total, but in Chapter IV, only preliminary results from the first sampling campaign are presented. However even if the modality with 16 species is missing, we were able to observe how the plant species richness impacts the AM fungal community. Our preliminary results show that AM taxa richness is affected by the plant richness; it tends to decrease when the plant species richness is reduced. This corroborates results found in other studies (Burrows & Pfleger, 2002; Alguacil *et al.,* 2012).

The study reported in Chapter IV should have implications in agricultural development. Under current regimes, extensive monocultures are largely used, potentially leading to decreases in AM fungal diversity and ecosystem productivity (Tilman, 1996b; van der Heijden *et al.,* 1998; Klironomos *et al.,* 2000).

We also show that the AM community composition changes according to changes in plant hosts, both in the occurrence and proportion of some AM symbionts. This suggests the possible existence of generalists and specialists. The results are explained by (i) a host-plant preference phenomenon, (ii) a preference for the functional group to which the plant belongs (C3 metabolism, C4 metabolism, forb or legume), (iii) a niche complementarity, (iv) differences in competitive level displayed by the AM fungi.

Here only the taxonomical diversity of plants and AM fungi was considered. However, different levels of diversity exist: (1) functional diversity, (2) species richness and evenness and (3) intraspecific diversity (Johnson *et al.,* 2012). We focused solely on AM fungal species richness and evenness. Functional diversity and intraspecific diversity are not addressed in this PhD thesis. The intraspecific diversity of AM fungi has been little studied mainly because of lack of knowledge about (i) life cycle, (ii) ploidy level, (iii) coenocytic spores and cells. AMF display a high degree of *intra* individual sequence polymorphism (Corradi & Bonfante, 2012) and the existence of recombinations or recombination-like events have been demonstrated (e.g. Vandenkoornhuyse *et al.*, 2001). One process which generates intraspecific variations is the fusion of hyphae from different

spores. In addition, the segregation of nuclei within the spores will differ depending on the host plant which would help to maintain the genetic diversity in AM fungi.

Very little is known about the factors regulating genetic diversity within a given symbiosis. Both biotic and abiotic factors are involved in this regulation: the host-plant species and functions, and environmental conditions like phosphorus availability are known to induce genetic changes in AMF isolates (Ehinger *et al.,* 2009). This increasing of intra-isolate genetic variation will lead to competition not only between closely related isolates but also within the same isolate (Ehinger *et al.*, 2009). This will lead to the selection of particular genotypes, which in turn act on plant diversity. In addition, between-species interactions and fitness feedback might explain the coexistence of plant and AM fungal diversity in ecosystems. Several questions arise at this point: firstly, what is the extent of intraspecific diversity within an AM fungal taxon; secondly, does this extent of intraspecific diversity vary between different AM fungal taxa? Thirdly, does this intraspecific diversity lead to a diversity of functional traits?

3. Integrating other organisms

I was interested in studying the relationship between plant diversity and fungal diversity. The molecular markers we used specifically targeted arbuscular mycorrhizal fungi. However, a given host plant can be colonized by AM fungi and microbial endophytes simultaneously. The diversity of these endophytic microorganisms is not well known and they are usually described as fungal endophytes. Research on endophytic microorganisms has mainly been performed on Poaceae but includes a large array of plants, even pioneer plants such as *Arabidopsis thaliana* (e.g. Qiang *et al.*, 2012). These endophytes live in symbiosis with plant roots, stems or leaves, and their behaviour is known to range from mutualistic to pathogenic (for review see Rodriguez *et al.*, 2009). Like AM fungi, they display various host ranges, and their effects are variable depending on the host species but also within a same host species (Vaz *et al.*, 2012). They rely on plants for their survival and nutrient supply. Endophytes can improve the competitiveness, biomass and growth of their host, depending on the host-plant species and environmental conditions, (Waller *et al.*, 2005; Aschehoug *et al.*, 2012), and can confer tolerance to various stresses such as salinity, disease resistance (e.g. Waller *et al.*, 2005), and herbivore-resistance (Brem & Leuchtmann, 2001; Afkhami & Rudgers, 2009). AM fungi and endophytes have reciprocal effects on each other and could potentially interact synergistically to influence host-plant

fitness (Larimer *et al.*, 2012; Aschehoug, 2012). AM fungi and endophytes can also compete for the resources provided by the host-plant (Larimer *et al.*, 2012). Liu *et al.* (2011) identified competition between an AM fungus and a fungal endophyte depending on the P resource supply and the C content of the plant, with a decrease in AM fungal colonization when P was higher and a decrease in endophytes when C was higher. Some endophytes can enhance AM fungal colonization, thus promoting the plant symbiosis with AM fungi (Vaz *et al.*, 2012), this effect being dependent on the AM fungal species. Co-infection of a plant with AM symbiont(s) and endophyte(s), leads to decreased colonization by both symbionts and, in particular conditions, the host-plant is unable to maintain the two kinds of symbionts, resulting in a decrease of AM fungal colonization (Larimer *et al.*, 2010). This underlines the importance of not restricting investigations to the AM fungi but to have a broader view of root colonizing microorganisms. Different questions arise when the whole symbiotic pool of a given host-plant is considered: (i) are there high- and low-quality partners in other types of symbionts? (ii) what are the functions of endophytic symbionts (iii) how is cooperation maintained when there is a suite of competing species types?

In the future, dedicated markers could be used to identify all endophytic microorganisms associated with plants. The relative diversities of Fungi, Bacteria and Archaea could be examined to determine possible positive and negative correlations within the symbiont community and to detect possible associations and competitions within the host-plant.

V. Arbuscular mycorrhizal symbiosis for a sustainable agriculture

Understanding the mechanisms governing the AM mutualism (e.g. taking into account the different strategies of control adopted by plant and AMF, as well as the reciprocal effects of plant diversity on AM fungi) is really important as they are crucial components for plant productivity, ecosystem functioning and maintenance of soil fertility (Tilman, 1996b; van der Heijden *et al.,* 1998; Klironomos *et al.,* 2000). Currently, food demand is increasing while agricultural productivity is stagnating (Koning *et al.*, 2007; Depetris Chauvin *et al.*, 2012). Conventional agricultural practices often disrupt the AM symbiosis due to application of fertilizers and intensive tillage. As this symbiosis is considered as a mutualism which helps to 'feed the world' it is essential to increase farming yields while limiting or even decreasing fertilizer inputs. Promoting and improving this symbiosis functioning in agricultural systems must be a priority. Some simple

measures presented in the chapter V can be applied: (i) the use of greater plant diversity, (ii) restricted or no tillage, (iii) selection of plants in poor soils, (iv) promotion of other mutualisms.

The inoculation of AM fungi directly in soil was suggested as a solution to restore poor soils (He & Nara, 2007; Douds *et al.,* 2012; Vosatka *et al.,* 2012). However, this is hardly generalizable (Hart & Trevors, 2005) as production of such high quantities of AM fungal inoculum seems impossible with present-day technology. Furthermore it was shown that native AM fungi perform better than inoculated fungi even if the latter are beneficial (Rowe *et al.,* 2007: Johnson *et al.,* 2010). It has also been shown that addition of non-native AM fungi disrupts the native AM fungal diversity (Koch *et al.,* 2011). Thus, the best solution is likely to promote the AM fungi already in their endemic soil.

Conclusion:

From this thesis, new knowledge on the AM symbiosis evolution and mechanisms of stability has been acquired. The first body of new insights is presented in Chapters I and II, showing that bilateral control of symbiosis, via a nutrient supply dependant on the cooperative quality of the partners contributes to maintaining cooperation. Another novel insight is the potential involvement of the host plant in the protection of its AM symbiont against fungivory, by providing toxic metabolites repelling fungivores. New knowledge has also been gained on the effect of a loss in plant diversity on the AM fungal community structure and diversity (Chapter IV). The database presented in Chapter III was built to help analyse the data obtained from this study. From these results, new ideas for AM symbiosis in agricultural management are discussed.

Empirical approaches to studying plant–microbe interactions are generally reductionist because of the complexity of these relationships. Little by little, we need now to increase the level of complexity of our experiments by studying multiple symbionts within individual host plants, and in multiple hosts to obtain a more comprehensive view of the AM symbiosis and its interactions within an ecosystem. It is now also important to focus on other symbionts colonizing the plants like bacteria and fungal endophytes. We need to determine if the same mechanisms of control of the symbiosis exist and if potential protection of these symbionts occurs. It is also necessary to know the consequences of changing plant diversity on these other symbiotic interactions. This will allow a more detailed picture of these mutualisms. With these new findings, it will be possible to have a better understanding of their role in regulating natural and agricultural ecosystems. In our changing planet, it is important to better understand and better use plant mutualisms to meet the future demand for foods.

References:

Abarenkov K. *et al.* 2010a. The UNITE database for molecular identification of fungi-recent updates and future perspectives. New Phytologist 186: 281-285

Abarenkov K. *et al.* 2010b. PlutoF-a Web Based Workbench for Ecological and Taxonomic Research, with an Online Implementation for Fungal ITS Sequences. Evol Bioinformatics 6: 189-196

Abdel-Fattah G.M. *et al.* 2011. Induction of defense responses in common bean plants by arbuscular mycorrhizal fungi. Microbiological Research 166: 268-281

Afkhami M.E. & Rudgers J.A. 2009. Endophyte-mediated resistance to herbivores depends on herbivore identity in the wild grass *Festuca subverticillata*. Environmental Entomology 38: 1086-95

Aitchison P.A. & Butt V.S. 1973. Relation between synthesis of inorganic polyphosphate and phosphate uptake by *Chlorella vulgaris*. Journal of Experimental Botany 24: 497-510

Akiyama K. *et al.* 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435:824-827

Albrecht C. *et al.* 1999. Legume nodulation and mycorrhizae formation, two extremes in host specificity meet. The EMBO Journal 18:281-288

Aldhous P. 2004. Land remediation: Borneo is burning. Nature 432: 144-146

Alguacil M. *et al.* 2012. Changes in the diversity of soil arbuscular mycorrhizal fungi after cultivation for biofuel production in a Guantanamo (Cuba) tropical system. PLoS ONE 7: e34887

Alkan N. *et al.* 2006. Analysis of quantitative interactions between two species of Arbuscular Mycorrhizal Fungi, *Glomus mossae* and *G. intraradices*, by real-time PCR. Applied and Environmental Microbiology 72: 4192-4199

Aly A.H. *et al.* 2010. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Diversity 41: 1-16

An G.H. *et al.* 2010 How does arbuscular mycorrhizal colonization vary with host plant genotype? An example based on maize (*Zea mays*) germplasms. Plant Soil 327 : 441–453

Anderson I.C. & Parkin P.I. 2007. Detection of active soil fungi by RT-PCR amplification of precursor rRNA molecules. Journal of Microbial Methodology 68: 248-253

Antunes P.M. *et al.* 2011. Evidence for functional divergence in arbuscular mycorrhizal fungi from contrasting climatic origins. New Phytologist 189: 507-2011

Archetti M. *et al.* 2011. Economic game theory for mutualism and cooperation. Ecology Letters 14: 1300-1312

St. Arnaud M. *et al.* 1996. Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. Mycological Research 100: 328-332

Arnon D.I. & Hoagland D.R. 1940. Crop production in artificial culture solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. Soil Science 50: 463-485

Aschehoug E.T. *et al.* 2012 Fungal endophytes directly increase the competitive effects of an invasive forb. Ecology 93: 3-8

Atul-Nayyar A. *et al.* 2009. The arbuscular mycorrhizal symbiosis links N mineralization to plant demand. Mycorrhiza 19: 239-246

Avise J.C. 2004. Molecular markers, natural history, and evolution. Sunderland, Massachusetts: Sinauer Associates

Azcón-Aguilar C. & Barea J.M. 1996. Arbuscular mycorrhizas and biological controlof soil-borne plant pathogens-An overview of the mechanisms involved. Mycorrhiza 6: 457-464

Bago B. *et al.* 2000. Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiology 124: 949-957

Bago B. *et al.* 2002. Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis. Plant Physiology 128: 108-124

Bago B. *et al.* 2003. Carbon export from arbuscular mycorrhizal roots involves the translocation of carbohydrate as well as lipid. Plant Physiology 131: 1496-1507

Bakonyi G. *et al.* 2002. Density-dependent regulation of arbuscular mycorrhiza by collembola. Functional Ecology 34: 661-664

Baldauf S.L. & Palmer J.D. 1993. Animals and fungi are each others closest relatives: congruent evidence from multiple proteins. Proceedings of the National Academy of Sciences of the United States of America 90: 11558-11562

Baldauf S.L. 1999. A search for the origins of Animals and Fungi: comparing and combining molecular data. American Naturalist 154: S178-S188

Baldauf S.L. *et al.* 2000. A kingdom-level phylogeny of eukaryotes based on combined protein data. Science 290: 972-977

Balmford A. *et al.* 2012. What conservationists need to know about farming. Proceeding of the Royal Society B 279: 2714-2724

Bar-Hen A. *et al.* 2008. Influence function for robust phylogenetic reconstructions. Molecular Biology & Evolution 25: 869-873

Barto E.K. *et al.* 2011. The fungal fast lane: Common mycorrhizal networks extend bioactive zones of allelochemicals in soils. PLoS ONE 6: e27195

Barton K.E. 2007. Early ontogenetic patterns in chemical defense in *Plantago* (Plantaginaceae): Genetic variation and trade-offs. American Journal of Botany 94: 56-66

Bascompte J. 2009. Mutualistic networks. Frontiers in ecology and the environment 7: 429-436

Bass D. *et al.* 2007 Yeast forms dominate fungal diversity in the deep oceans. Proceeding of the Royal Society B 274: 3069-3077

Battisti D.S. & Naylor R.L. 2009. Historical warnings of future food insecurity with unprecedented seasonal heat. Science 323: 240-244

Bennet A.J. *et al.* 2011. Meeting the demand for crop production: the challenge of yield decline in crops grown in short rotations. Biological Reviews 87: 52–71

Benson D.A. *et al.* 2011. GenBank. Nucleic Acids Research 39: D32-D37

Bever J.D. *et al.* 1996. Host-de-pendent sporulation and species diversity of arbuscular mycorrhizal fungi in a mown grassland. Journal of Ecology 84: 71-82

Bever J.D. *et al.* 2009. Preferential allocation to beneficial symbiont with spatial structure maintains mycorrhizal mutualism. Ecology Letters 12: 13-21

Bhattacharya D. *et al.* 2002. Vertical evolution and intragenic spread of lichen-fungal group I

intron. Journal of Molecular Evolution 55: 74-84

Bhattacharjee S. & Sharma G.D. 2012. The vesicular arbuscular mycorrhiza associated with three cultivars of rice (*Oryza sativa L*.). Indian Journal of Microbiology 51: 377-383

Bidartondo M.I. 2008. Preserving accuracy in GenBank. Science 319: 1616

Biere A. *et al.* 2004. Plant chemical defense against herbivores and pathogens: generalized defense or trade offs? Oecologia 140: 430-441

Black K.G. *et al.* 2000. Effect of mycorrhizal-enhanced leaf phosphate status on carbon partitioning, translocation and photosynthesis in cucumber. Plant, Cell & Environment 23 *:* 797-809

Blackwell M. 2011. The Fungi: 1, 2, 3 … 5.1 million species? American Journal of Botany 98: 426-438

Blee K.A. & Anderson A.J. 1998. Regulation of arbuscule formation by carbon in the plant. Plant Journal 16: 523-530

Boehm A.M. *et al.* 2012. FoxO is a critical regulator of stem cell maintenance in immortal Hydra. Proceeding of the National Academy of Sciences USA 109: 19697-19702

Böllmann J. *et al.* 2010. Defensive strategies of soil fungi to prevent grazing by *Folsomia candida* (Collembola). Pedobiologia 53: 107-114

Bonfante P. & Genre A. 2010. Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. Nature Communications 48:1-11

Boon E. *et al.* 2010. Intra-isolate genome variation in arbuscular mycorrhizal fungi persists in the transcriptome. Journal of Evolutionary Biology 23: 1519-1527

Börstler B. *et al.* 2008. Genetic diversity of the arbuscular mycorrhizal fungus Glomus intraradices as determined by mitochondrial large subunit rRNA gene sequences is considerably higher than previously expected. New Phytologist 180: 452-465

Bowers M.D. & Puttick G.M. 1988. Response of generalist and specialist insects to qualititative allelochemical variation. Journal of Chemical Ecology 14: 319-334

Bowers M.D. *et al.* 1992. Effects of genotype, habitat, and seasonal variation on iridoid glycoside content of *Plantago lanceolata* (Plantaginaceae) and the implications for insect herbivores. Oecologia 91: 201-207

Brem D. & Leuchtmann A. 2001. *Epichloe* grass endophytes increase herbivore resistance in the woodland grass *Brachypodium sylvaticum*. Oecologia 126: 522-530

Breuillin F. *et al.* 2002. Phosphate systemically inhibits development of arbuscular mycorrhiza in *Petunia hybrida* and represses genes involved in mycorrhizal functioning. Plant Journal 64: 1002-1017

Bridge P.D. *et al.* 2003. On the unreliability of published DNA sequences. New Phytologist 160: 43-48

Bridge P.D. *et al.* 2004. Reliability and use of published sequence data. New Phytologist 161: 15-17

Bright M. & Bulgheresi S. 2010. A complex journey: transmission of microbial symbionts. Nature Review Microbiology 8:218-230

Brown *et al.* 2001. Complex species interactions and the dynamics of ecological systems: Long-term experiments. Science 293: 643-65

Brundrett M.C. 2009. Mycorrhizal associations and other means of nutrition of vascular plants:

understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant and Soil 320:37-77

Bshary A. & Noë R. 2003 in Genetic and Cultural Evolution of Cooperation, P. Hammerstein, Ed. (MIT Press, Cambridge) 167-184

Bucher M. 2007. Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. New Phytologist 173: 11-26

Bucher M. *et al.* 2009. Chasing the structures of small molecules in arbuscular mycorrhizal signaling. Current Opinion in Plant Biology 12: 500-507

Bücking H. & Shachar-Hill Y. 2005. Phosphate uptake, transport and transfer by the arbuscular mycorrhizal fungus *Glomus intraradices* is stimulated by increased carbohydrate availability. New Phytologist 165: 899-912

Burney J.A. *et al.* 2010. Greenhouse gas mitigation by agricultural intensification. Proceedings of the National Academy of Sciences of the United States of America 107:12052-12057

Burrows R.L. & Pfleger F.L. 2002. Arbuscular mycorrhizal fungi respond to increasing plant diversity. Canadian Journal of Botany 80: 120-130

Callaway R.M. *et al.* 2008. Novel weapons: invasive plant suppresses fungal mutualists in America but not in its native Europe. Ecology 89:1043-1055

Cameron D.D. *et al.* 2008. Giving and receiving: measuring the carbon cost of mycorrhizas in the green orchid*, Goodyera repens*. New Phytologist 180: 176-184

Cano C. *et al.* 2009. *Glomus custos sp nov*., isolated from a naturally heavy metal-polluted environment in southern Spain. Mycotaxon 109: 499-515

Cavalier-Smith T. 2002. The phagotrophic origin of eukaryotes andphylogenetic classification of Protozoa. International Journal of Systematic and Evolutionary Microbiology 52: 297-354

Chabot S. *et al.* 1992. Life cycle of *Glomus intraradix* in root organ culture. Mycologia 84: 315-321

Chauvin N.D. *et al.* 2012. Food Production and Consumption Trends in Sub-Saharan Africa: Prospects for the Transformation of the Agricultural Sector. Working Paper, United Nations Development Program WP 2012-011:1-74

Choi I.G. & Kim S.H. 2007. Global extent of horizontal gene transfer. Proceedings of the National Academy of Sciences of the United States of America 104: 4489-4494

Cienkowski L. 1871. Über Scwarmer-Bildung bei Radiolarien. Archiv für Mikroskopische Anatomie 7: 372-381

Cirera X. & Masset E. 2010. Income distribution trends and future food demand. Philosophical Transactions of the Royal Society B 365: 2821-2834

Clapp J.P. *et al.* 2001. Inter-and intra-isolate rRNA large subunit variation in *Glomus coronatum* spores. New Phytologist 149: 539-554

Cole J.R. *et al.* 2009. The Ribosomal Database Project: Improved alignments and new tools for rRNA analysis. Nucleic Acids Research 37: D141-D145

Cordell D. *et al.* 2009. The story of phosphorus, global food security and food for thought. Global Environmental Changes 19: 292-305

Corradi N. & Bonfante P. 2012. The arbuscular mycorrhizal symbiosis: Origin and evolution of a beneficial plant infection. PLoS Pathogen 8: e1002600

Cowden C.C. & Peterson C.J. 2009. A multi-mutualist simulation: Applying biological market models to diverse mycorrhizal communities. Ecological Modelling 220: 1522-1533

D'Auria G. *et al.* 2006. IWoCS analyzing ribosomal intergenic transcribed spacers configuration and taxonomic relationships. Bioinformatics 22: 527-531

Darrow K. Bowers M.D. 1997. Phenological and population variation in iridoid glycosides of *Plantago lanceolata* (Plantaginaceae). Biochemical Systematics and Ecology 25: 1-11

Darrow K. & Bowers M.D. 1999. Effects of herbivore damage and nutrient level on induction of iridoid glycosides *in Plantago lanceolata*. Journal of Chemical Ecology 25: 1427-1440

Davitt A.J. *et al.* 2010. Do the costs and benefits of fungal endophyte symbiosis vary with light availability? New Phytologist 188:824-834

De Bary A. 1879. "Die Erscheinung der Symbiose". Privately printed in Strasburg

De Deyn G.B. *et al.* 2009. Chemical defense, mycorrhizal colonization and growth responses *in Plantago lanceolata L*. Oecologia 160: 433-442

De Santis T.Z. *et al.* 2006. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. Applied & Environmental Microbiology 72: 5069-5072

De Souza Andrade D. *et al.* 2003. Long-term effects of agricultural practices on microbial community. In Conservation agriculture. (García-Torres, L. *et al.* eds.), pp. 301-306 Kluwer Academic Publishers, Dordrecht, Netherlands

Denison R. F. *et al.* 2003. Ecology. The Ecological Society of America. 84:838-845

Dinda B. *et al.* 2007. Naturally occurring iridoids: A review, Part 1. Chemical and Pharmaceutical Bulletin 55: 159-227

Doner L.W. & Becard G. 1991. Solubilization of gellan gels by chelation of cations. Biotechnology Techniques 5: 25-28

Douds D.D. *et al.* 2012. Utilization of inoculum of AM fungi produced on-farm for the production of *Capsicum annuum*: A summary of seven years of field trials on a conventional vegetable farm. Biological Agriculture and Horticulture 28: 129-145

Douglas A.E. 2008. Conflict, cheats and persistence of symbioses. New Phytologist 177: 849-858

Douglas A.E. 2010. The Symbiotic Habit. (Princeton Univ. Press., Princeton, NJ)

Drigo B. *et al.* 2010. Shifting carbon flow from roots into associated microbial communities in response to elevated atmospheric CO2. Proceedings of the National Academy of Sciences of the United States of America 107: 10938-10943

Egerton-Warburton L.M. *et al.* 2007. Mycorrhizal community dynamics following nitrogen fertilization, a cross-site test in five grasslands. Ecological Monographs 77: 527-544

Egger K.N. & Hibbett D.S. 2004. The evolutionary implications of exploitation in mycorrhizas. Canadian Journal of Botany 82: 1110-1121

Ehinger M. *et al.* 2009. Changes in arbuscularmycorrhizal fungal phenotypes and genotypes in response to plant species identity and phosphorus concentration. New Phytologist 184: 412-423

Elser J. & Bennett E. 2011. A broken biogeochemical cycle. Nature 478:29-31

Eom A.H. *et al.* 1999. The effect of fire, mowing and fertilizer amendment on arbuscular mycorrhizas in tallgrass prairie. American Midland Naturalist 142: 55-70

Fajer E.D. *et al.* 1992. The effect of nutrients and enriched CO2 environments on production of carbon-based allelochemicals in *Plantago*: A test of the carbon/nutrient balance hypothesis. The American Naturalist 140: 707-723

Fellbaum C.R. *et al.* 2012. Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis. Proceedings of the National Academy of Sciences of the United States of America 109: 2666-2671

Felsenstein J. 2004. Inferring phylogenies. Sinauer Associates, Sunderland, Massachusetts

Ferrol N. *et al.* 2002. Mechanisms of nutrient transport across interfaces in arbuscular mycorrhizas. Plant Soil 244: 231-237

Field B. *et al.* 2006. Tansley review: First encounters-deployment of defence-related natural products by plants. New Phytologist 172: 193-207

Fitter A.H. 1991. Costs and benefits of mycorrhizas: implications for functioning under natural conditions. Experientia 47: 350-355

Fitter A.H. 2006. Carbon and phosphorus fluxes in arbuscular mycorrhizas? A null hypothesis for symbiotic function. New Phytologist 172:3-6

Fitter A.H. *et al.* 2011. Nutritional exchanges in the arbuscular mycorrhizal symbiosis: implications for sustainable agriculture. Fungal Biology Reviews 25: 68-72

Fleetwood D. J. *et al.* 2007. A complex ergovaline gene cluster in *Epichloe* endophytes of grasses. Applied and Environmental Microbiology 73: 2571-2579

Foley J.A. *et al.* 2005. Global consequences of land use. Science 309: 570-574

Foley J.A. *et al.* 2011. Solution for a cultivated planet. Nature 478: 337-342

Fontana A. *et al.* 2009. The effects of arbuscular mycorrhizal fungi on direct and indirect defense metabolites *of Plantago lanceolata L*. Journal of Chemical Ecology 35: 833-43

Fortin J.A. *et al.* 2002. Arbuscular mycorrhiza on root-organ cultures. Canadian Journal of Botany 80:1-20

Fountain M.T. & Hopkin S.P. 2005. *Folsomia candida* (Collembola): a “standard” soil arthropod. Annual Review of Entomology 50: 201-22

Formey D. *et al.* 2012. Comparative analysis of mitochondrial genomes of *Rhizophagus irregularis* – syn. *Glomus irregulare* - reveals a polymorphism induced by variability generating elements. New Phytologist 196: 1217-1227

Frank A.B. 1877. Über die biologischen Verhältnisse des Thallus einiger Krustenflechten.- Beiträge zur Biologie der Pflanzen 2: 123-200

Frank A. 1996. Host-symbiont conflict over the mixing of symbiotic lineages. Proceedings of the National Academy of Sciences of the United States of America 263: 339-344

Franz B.F. & Hijri M. 2009. The complete *Glomus intraradices* mitochondrial genome sequence - a milestone in mycorrhizal research. New Phytologist 183:4-6

Frederickson M.E. *et al.* 2012. The direct and ecological costs of an ant-plant symbiosis. The

American Naturalist 179: 768-778

Fuchs A. & Bowers M.D. 2004. Patterns of iridoid glycoside production and induction in *Plantago lanceolata* and the importance of plant age. Journal of Chemical Ecology 30: 1723-1724

Gadkar V. *et al.* 2003. Root exudate of pmi tomato mutant M161 reduces AM fungal proliferation in vitro. FEMS Microbiology Letters 223: 193-198

Gange A.C. & West H.M. 1994. Interactions between arbuscular mycorrhizal fungi and foliar-feeding insects in *Plantago lanceolata* L. New Phytologist 128: 79-87

Gange A.C. 2000. Arbuscular mycorrhizal fungi, Collembola and plant growth. Trends of Ecological Evolution 15: 369-372

Genre A. *et al.* 2005. Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. Plant Cell 17: 3489-3499

Gerdemann J.W. & Trappe J.M. 1974. The Endogonaceae in the Pacific North-West. Mycological Memoirs 5: 1-76

Gerdemann J.W. & Nicolson T.N. 1962. Endogone spores in cultivated soils. Nature 195: 308-309

Gianinazzi-Pearson V. 1984. Host-fungus specificity, recognition and compatibility in mycorrhizae. In: "Genes Involved In Microbe Plant Interactions." Advances In Plant Gene Research, Basic Knowledge and Application , Dennis E.S., Hohn B., Hohn T., King P., Schell I., Varma D.P.S. (eds)., Springer Verlag, Vienna and New York, 225-253

Gigante Carvalheiro L. *et al.* 2011. Natural and within-farmland biodiversity enhances crop productivity. Ecology Letters 14:251-259

Giller P.S. 1996. The diversity of soil communities, the “poor man’s tropical rainforest”. Biodiversity and Conservation 5:135-168

Giovannetti M. *et al.* 1993. Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. New Phytologist 125:587-593

Giovannetti M. *et al.* 1994. Early processes involved in host recognition by arbuscular mycorrhizal fungi. New Phytologist 127: 703-709

Golubski A.J. & Klausmeier C.A. 2010. Control in mutualisms: Combined implications of partner choice and bargaining roles. Journal of Theoretical Biology 267: 535 545

Gorissen A. *et al.* 1996. ESPAS-An advanced phytotron for measuring carbon dynamics in a whole plant-soil system. Plant Soil 179: 81-87

Goto R. *et al.* 2010. Selective flower abortion maintains moth cooperation in a newly discovered pollination mutualism. Ecology Letters 13: 321

Govindarajulu M. *et al.* 2005. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435*:* 819-823

Grime J.P. *et al.*1987. Floristic diversity in a model system using experimental microcosms. Nature 328:420-422

Guindon S. *et al*. 2010. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. Systematic Biology 59:307-321

Gyuricza V. *et al.* 2010. Radiocesium transfer between Medicago truncatula plants via a common mycorrhizal network. Environmental Microbiology 12*:* 20180-20189

Hammer E.C. *et al.* 2011. Elemental composition of arbuscular mycorrhizal fungi at high salinity. Mycorrhiza 21: 117-129

Hardin G. 1968. The Tragedy of the Commons. Science162:1243-1248

Harris K.K & Boerner R.E.J. 1990. Effects of belowground grazing by collembola on growth, mycorrhizal infection, and P uptake of *Geranium-robertianum*. Plant and Soil 129: 203-210

Harrison M.J. *et al.* 2002. A phosphate transporter from *Medicago truncatula* involved in the acquisiton of phosphate released by arbuscular mycorrhizal fungi. Plant Cell 14: 2413-2429

Hart M.M & Reader R.J. 2002. Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytologist 153: 335-344

Hart M.M. *et al.* 2003. Plant coexistence mediated by arbuscular mycorrhizal fungi. Trends in Ecology and Evolution 18: 418-423

Hart M.M. & Reader R.J. 2005. The role of the external mycelium in early colonization for three arbuscular mycorrhizal fungal species with different colonization strategies. Pedobiologia 49: 269-279

Hart M.M. & Jack T.T. 2005. Microbe management: application of mycorrhyzal fungi in sustainable agriculture. Frontiers in Ecology & Environment 3: 533–539

Harte J. 2007. Human population as a dynamic factor in environmental degradation. Population & Environment 28: 223-236

Hartmann A. *et al.* 2009. Plant-driven selection of microbes. Plant Soil 321:235-257

Hausmann N.T. & Hawkes C.V. 2009. Plant neighborhood control of arbuscular mycorrhizal community composition. New Phytologist 183:1188-1200

Hausmann N.T. & Hawkes C.V. 2010. Order of plant host establishment alters the composition of arbuscular mycorrhizal communities. Ecology 91: 2333-2343

Hawkes C.V. *et al.* 2006. Arbuscular mycorrhizal assemblages in native plant roots change in the presence of invasive exotic grasses. Plant Soil 281:369-380

Hawksworth D.L. & Rossman A.Y. 1997. Where are all the undescribed fungi? Phytopathology 87: 888-891

Hawksworth D.L. 2004. 'Misidentification' in fungal DNA sequence databanks. New Phytologist 161: 13-15

Hawksworth D.L. 2009. Separate name for fungus's sexual stage may cause confusion. Nature 458: 29

He X. & Nara K. 2007. Element biofortification: can mycorrhizas potentially offer a more effective ans sustainable pathway to curb human malnutrition ? Trends in Plant Science 12: 331-333

Hector A. *et al*. 2000. Plant diversity and productivity experiments in European grasslands. Science 286: 1123-1127

Helgason T. *et al.* 1998. Ploughing up the wood-wide web? Nature 394:431

Helgason T. 2002. Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland. Journal of Ecology 90: 371-384

Helgason T. *et al.* 2003. Phylogeny of the Glomerales and Diversisporales (Fungi: Glomeromycota) from actin and elongation factor 1-alpha sequences. FEMS

Microbiology Letters 229: 127-132

Helgason T. 2007. Specificityand resilience in the arbuscular mycorrhizal fungi of a natural woodland community. Journal of Ecology 95: 623-630

Helgason T. & Fitter A.H. 2009. Natural selection and the evolutionary ecology of the arbuscular mycorrhizal fungi (Phylum Glomeromycota). Journal of Experimental Botany 60: 2465-2480

Hibbett D.S. *et al.* 2007. A higher-level phylogenetic classification of the Fungi. Mycological Research 111: 509-547

Hibbett D.S. 2011. Progress in molecular and morphological taxon discovery of Fungi and options for formal classification of environmental sequences. Fungal Biology Reviews 25: 38-47

Hishi T. *et al.* 2008. Relationships among fine roots, fungal hyphae and soil microarthropods among different soil microhabitats in a temperate coniferous forest of *Chamaecyparis obtusa*. European Journal of Soil Biology 44: 473-477

Hoagland D.R. & Arnon D.I. 1950. The water-culture method for growing plants without soil. Circ. 347. University of Californy Agricultural Experimental Station, Berkley

Hodge A. *et al.* 2010. Nutritional ecology of arbuscular mycorrhizal fungi. Fungal Ecology 3: 267-273

Hodge A. & Fitter A.H. 2010. Substantial nitrogen acquisition by arbuscular mycorrhizal fungi from organic material has implications for N cycling. Proceedings of the National Academy of Sciences of the United States of America 107: 13754-13759

Hoeksema J.D. & Kummel M. 2003. Ecological persistence of the plant-mycorrhizal mutualism: A hypothesis from species coexistence theory. American Naturalist 162 (Suppl), S40

Hoeksema J.D. *et al.* 2010. A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. Ecology Letters 13: 394-407

Holst-Jensen A. *et al.* 2004. On reliability. New Phytologist 161: 11-13

Hooper D.U. *et al.* 2005. Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecological Monographs 75: 3–35

Horrigan L. *et al.* 2002. How sustainable agriculture can address the environmental and human. Health Harms of Industrial Agriculture 110: 445-456

Hughes A.R. *et al.* 2008. Ecological consequences of genetic diversity. Ecology Letters 11: 609-623

Humphreys C.P. *et al.* 2010. Mutualistic mycorrhiza-like symbiosis in the most ancient group of land plants. Nature Communications 1: 103

Husband R. 2002. Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. Molecular Ecology 11: 2669-2678

Jaafar Z. & Hou Z. 2012. Partner choice in gobiid fish *Myersina macrostoma* living in association with the alpheid shrimp *Alpheus rapax*. Symbiosis 56: 121-127

Jandér K.C. & Herre E.A. 2010. Host sanctions and pollinator cheating in the fig tree-fig wasp mutualism. Proceeding of the Royal Society B 277: 1481-1488

Jansa J. *et al.* 2005. Phosphorus acquisition strategies within arbuscular mycorrhizal fungal community of a single field site. Plant & Soil 276: 163-176

Jansa J. *et al.* 2008. Are there benefits of simultaneous root colonization by different

arbuscular mycorrhizal fungi? New Phytologist 177: 779-789

Jastrow J.D. *et al.* 1998. Contributions of interacting biological mechanisms to soil aggregate stabilization in restored prairie. Soil Biology & Biochemistry 30: 905-916

Javaid A. 2007. Allelopathic interactions in mycorrhizal associations. Allelopathy Journal 20: 29-42

Javot H. *et al.* 2007a. A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. Proceedings of the National Academy of Sciences of the United States of America 104: 1720-1725

Javot H. *et al.* 2007b. Phosphate in the arbuscular mycorrhizal symbiosis: transport properties and regulatory roles. Plant Cell & Environment 30: 310-322

Jin H. *et al.* 2005. The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis. New Phytologist 168: 687-696

Johnson N.C. *et al.* 1992. Mycorrhizae-possible explanation for yield decline with continuous corn and soybean. Agronomical Journal 84: 387-390

Johnson N.C. 1993. Can fertilization of soil select less mutualistic mycorrhizae. Ecological Applications 3: 749-757

Johnson N.C. *et al.* 1997. Functioning of mycorrhizas along the mutualism-parasitism continuum. New Phytologist 135: 1-12

Johnson D *et al.* 2001. Novel in-growth core system enables functional studies of grassland mycorrhizal mycelial networks. New Phytologist 162: 555-562

Johnson N.C. *et al.* 2003a. Interactions among mycorrhizae, atmospheric CO2 and soil N impact plant community composition. Ecology Letters 6: 532-540

Johnson N.C. *et al.* 2003. Nitrogen enrichment alters mycorrhizal allocation at five mesic to semiarid grasslands. Ecology 84: 1895-1908

Johnson D. *et al.* 2004. Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosms. New Phytologist 161:503-515

Johnson D. *et al.* 2005. Soil invertebrates disrupt carbon flow through fungal networks. Science 309: 1047-1047

Johnson N.C. & Gehring C.A. 2007. Mycorrhizas symbiotic mediators of rhizosphere and ecosystems processes in the Rhizosphere: an ecological perspective. In Z. Cardon and J. Whitbeck (eds). The Rhizosphere-an Ecological Perspective. Academic Press

Johnson N.C. *et al.* 2010. Resource limitation is a driver of local adaptation in mycorrhizal symbioses. Proceedings of the National Academy of Sciences of the United States of America 107: 2093-2098

Johnson D. *et al.* 2010. Plant genotypic diversity does not beget root-fungal species diversity. Plant and Soil 336: 107-111

Johnson D. *et al.* 2012. The importance of individuals: intraspecific diversity of mycorrhizal plants and fungi in ecosystems. New Phytologist 194: 614-628

Jonas J.L. *et al.* 2007. Consumption of mycorrhizal and saprophytic fungi by Collembola in grassland soils. Soil Biology and Biochemistry 39: 2594-2602

Jones M.D. & Smith S.E. 2004. Exploring functional definitions of mycorrhizas: Are mycorrhizas always mutualisms? Canadian Journal of Botany 82: 1089-1109

Jones M.D.M. *et al.* 2011. Discovery of novel intermediate forms redefines the fungal tree of

life. Nature 474: 200-203

Jørgensen H.B. *et al.* 2005. Selective foraging of fungi by collembolans in soil. Biology Letters 1: 243-246

Jung S.C. *et al.* 2012. Mycorrhiza-induced resistance and priming of plant defenses. Journal of Chemical Ecology 38: 651-664

Kaminuma E. *et al.* 2011. DDBJ progress report. Nucleic Acids Research 39: D22-D27

Kaschuk G. *et al.* 2009. Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? Soil Biology and Biochemistry 41: 1233-1244

Kempken F. & Rohlfs M. 2010. Fungal secondary metabolite biosynthesis-a chemical defence strategy against antagonistic animals? Fungal Ecology 3: 107-114

Kiers E.T. *et al.* 2003. Host sanctions and the legume-rhizobium mutualism. Nature 425: 78-81

Kiers E.T. & van der Heijden M.G.A. 2006 Mutualistic stability in the arbuscular mycorrhizal symbiosis, exploring hypotheses of evolutionary cooperation. Ecology 87: 1627-1636

Kiers E.T. *et al.* 2007. Human selection and the relaxation of legume defences against ineffective rhizobia. Proceedings of the National Academy of Sciences of the United States of America 274:3119-3126

Kiers E.T. & Denison R. F. 2008. Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. Annual Review of Ecology, Evolution, and Systematics 39: 215-236

Kiers E.T. *et al.* 2010. Mutualisms in a changing world: an evolutionary perspective. Ecology Letters 13: 1459-1474

Kiers E.T. *et al.* 2011. Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. Science 333: 880-882

Klironomos J.N. & Kendrick W.B. 1996. Palatability of microfungi to soil arthropods in relation to the functioning of arbuscular mycorrhizae. Biology and Fertility of Soils 21: 43-52

Klironomos J.N. & Ursic M. 1998. Density-dependent grazing on the extraradical hyphal network of the arbuscular mycorrhizal fungus, *Glomus intraradices*, by the collembolan, *Folsomia candida*. Nature 396: 69-72

Klironomos J.N. & Moutoglis P. 1999. Colonization of nonmycorrhizal plants by mycorrhizal neighbours as influenced by the collembolan, *Folsomia candida*. Biology and Fertility of Soils 29: 277-281

Klironomos J.N. *et al.* 2000. The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. Ecology Letters 3: 137-141

Klironomos J.N. 2002. Feedback with soil biota contributes toplant rarity and invasiveness in communities. Nature 417:67-70

Kobae Y. & Hata S. 2010. Dynamics of periarbuscular membranes visualized with a fluorescent phosphate transporter in arbuscular mycorrhizal roots of rice. Plant & Cell Physiology 51: 341-353

Koch A.M. *et al.* 2006. Genetic variability in a population of arbuscular mycorrhizal fungi causes variation in plant growth. Ecology Letters 9: 103-110

Koch A.M. *et al.* 2011. The effects of arbuscular mycorrhizal (AM) fungal and garlic mustard

introductions on native AM fungal diversity. Biological Invasions 13:1627-1639

Koga R. *et al.* 2012. Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. Proceedings of the National Academy of Sciences of the United States of America 109: E1230-E1237

Koide R.T. & Dickie I.A. 2002. Effects of mycorrhizal fungi on plant populations. Plant and Soil 244:307-317

Kõljalg U. *et al.* 2005. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytologist 166: 1063-1068

Koning N.B.J. *et al.* 2008. Long-term global availability of food : continued abundance or new scarcity. NJAS Wageningen Journal of Life Sciences 55: 229-292

Kuhn J. *et al.* 2004. Selective transport systems mediate sequestration of plant glucosides in leef beetles: A molecular basis for adaptation and evolution. Proceedings of the National Academy of Sciences of the United States of America 101: 13808-13813

Kulikova T. *et al.* 2007. The EMBL nucleotide sequence database in 2006. Nucleic Acids Research 35: D16-D20

Larimer A.L. *et al.* 2010. The interactive effects of plant microbial symbionts: a review and meta-analysis. Symbiosis 51*:*139-148

Larimer A.L. *et al.* 2012. Consequences of simultaneous interactions of fungal endophytes and arbuscular mycorrhizal fungi with a shared host grass. Oïkos 121*:*2090-2096

Larkin M.A. *et al.* 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948

Larsen J. *et al.* 2008. Population performance of collembolans feeding on soil fungi from different ecological niches. Soil Biology & Biochemistry 40: 360-369

Le Calvez T. *et al.* 2009. Fungal diversity in deep-sea hydrothermal ecosystems. Applied Environmental Microbiology 75: 6415-6421

Leake J.R. *et al.* 2004. Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. Canadian Journal of Botany 82:1016-1045

Leigh E.G. 2010. The evolution of mutualism. Journal of Evolutionary Biology 23*:* 2507-2528

Lekberd Y. & Koide R.T. 2005. Is plant performance limited by abundance of arbuscular mycorrhizal fungi? A meta-analysis of studies published between 1988 and 2003. New Phytologist 168*:*189-204

Lekberg Y. *et al.* 2010. Plants as resource islands and storage units-adopting the mycocentric view of arbuscular mycorrhizal networks. FEMS Microbiology Ecology 74*:* 336-345

Lekberg Y. *et al*. 2012. 454-sequencing reveals stochastic local reassembly and high disturbance tolerance within arbuscular mycorrhizal fungal communities. Journal of Ecology 100 : 151-160

Lewis C.L. & Coffroth M.A. 2004. The Acquisition of exogenous algal symbionts by an octocoral after bleaching. Science 304:1490-1492

Lilienfein J. *et al.* 2000. Soil acidification in Pinus caribaea forests on Brazilian savanna Oxisols Forest Ecology & Management 128: 145-157

Lim Y.W. *et al.* 2008. Distinguishing homokaryons and heterokaryons in *Phellinus sulphurascens* using pairing tests and ITS polymorphisms. Antonie van Leeuwenhoek 93: 99-110

Lin X.J. *et al.* 2012. Long-term balanced fertilization decreases arbuscular mycorrhizal fungal

diversity in an arable soil in North China Revealed by 454 pyrosequencing. Environmental science and technology 46:5764-5771

Liu Q. *et al*. 2011. Competition between foliar *Neotyphodium lolii* endophytes and mycorrhizal *Glomus spp.* fungi in *Lolium perenne* depends on resource supply and host carbohydrate content. Functionnal Ecology 25 : 910-920

Loreau M. & Hector A. 2001. Partitioning selection and complementarity in biodiversity experiments. Nature 412: 72-76

Lutz W. *et al.* 2001. The end of world population growth. Nature 412: 543-545

Lutzoni F. *et al.* 2004. Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. American Journal of Botany 91: 1446-1480

Mahe S. *et al.* 2012. PHYMYCO-DB: A Curated Database for Analyses of Fungal Diversity and Evolution. PloS One 7: e43117

Maherali H. & Klironomos J.N. 2007. Influence of Phylogeny on fungal community assembly and ecosystem functioning. Science 316: 1746-1748

Maier W. *et al.* 1995. Levels of a terpenoid glycoside (blumenin) and cell wall-bound phenolics in some cereal mycorrhizas. Plant Physiology 109: 465-470

Manefield M. *et al.* 2002. RNA stable isotope probing, a novel means of linking microbial community function to Phylogeny. Applied & Environmental Microbiology 68: 5367-5373

Marak H.B. *et al.* 2000. Direct and correlated responses to selection on iridoid glycosides in *Plantago lanceolata* L. Journal of Evolutionary Biology 13: 985-996

Marak H.B. *et al.* 2002. Two herbivore-deterrent iridoid glycosides reduce the in vitro growth of a specialist but not of a generalist pathogenic fungus of *Plantago lanceolata* L. Chemoecology 12: 185-192

Margulis L. 1992. Biodiversity: molecular biological domains, symbiosis, and kingdom origins. BioSystems 27: 39-51

Margulis L. 1993. From Gaia to Microcosm. BioSystems 31: 83

Martínez-García L.B. *et al.* 2011. Shrubs influence arbuscular mycorrhizal fungi communities in a semiarid environment. Soil Bioogy and Biochemistry 43: 682-689

Martinez-Garcia L.B. & Pugnaire F.I. 2011. Arbuscular mycorrhizal fungi host preference and site effects in two plant species in a semiarid environment. Applied Soil Ecology 48: 313-317

Marx J. 2004. The roots of plant-microbe collaborations. Science 304: 234-236

de Mazancourt D. & Schwartz M.W. 2010. A resource ratio theory of cooperation. Ecology Letters 13: 349-359

McCutcheon J.P. & Moran N.A. 2012. Extreme genome reduction in symbiotic bacteria. Nature Reviews Microbiology 10: 13-26

McGonigle T.P. *et al.* 1990. A new method which gives an objective measure of colonization of roots by vesicular arbuscular mycorrhizal fungi. New Phytologist 115: 495-501

Mikkelsen B.L. *et al.* 2008. Underground resource allocation between individual networks of mycorrhizal fungi. New Phytologist 180: 890-898

Mishler B.D. & Brandon R.N. 1987. Individuality, pluralism, and the phylogenetic species

concept. Biology and Philosophy 2: 397-414

MØller C.L. *et al.* 2013. Organic enrichment of sediments reduces arbuscular mycorrhizal fungi in oligotrophic lake plants. Freshwater Biology 58: 769-779

Moon C.D. *et al.* 2002. The evolutionary origins of three new *Neotyphodium* endophyte species from grasses indigenous to the Southern Hemisphere. Mycologia 94: 694-711

Moore J. C. *et al.* 1984. Effects of different management practices on Collembola and *Acarina* in corn production systems. The effects of no-tillage and Atrazine. Pedobiologia 26: 143-152

Moora M. *et al*. 2004. Native arbuscular mycorrhizal fungal communities differentially influence the seedling performance of rare and common *Pulsatilla* species. Functionnal Ecology 18:554-562

Morton J.B. & Benny G.L. 1990. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. Mycotaxon 37: 471-491

Nagy R. *et al.* 2009. Mycorrhizal phosphate uptake pathway in tomato is phosphorus-repressible and transcriptionally regulated. New Phytologist 181: 950-959

Nelson E. *et al.* 2010. Projecting global land-use change and its effect on ecosystem service provision and biodiversity with simple models. PloS ONE 5: e14327

Nilsson R.H. *et al.* 2006. Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. PloS One 1: e59

Noda S. *et al.* 2007. Cospeciation in the triplex symbiosis of termite gut protists (*Pseudotrichonympha spp*.), their hosts, and their bacterial endosymbionts. Molecular Ecology 16: 1257-1266

Noë R. & Hammerstein P. 1995. Biological markets. Trends in Ecology & Evolution 10: 336-339

Thiéry O. 2010. Molecular markers from the mitochondrial genome of arbuscular mycorrhizal fungi (Glomeromycota): evolutionary dynamics and application. PhD Thesis, University of Basel. Faculty of Science

Oehl F. *et al.* 2011. Glomeromycota: three new genera, and glomoid species reorganized. Mycotaxon 116: 75-120

Ohtomo R. & Saito M. 2005. Polyphosphate dynamics in mycorrhizal roots during colonization of an arbuscular mycorrhizal fungus. New Phytologist 167: 571-578

Olsson P.A. *et al.* 2002. Phosphorus effects on metabolic processes in monoxenic arbuscular mycorrhiza cultures. Plant Physiology 130*:* 1162-1171

Olsson P.A & Johnson N.C. 2005. Tracking carbon from the atmosphere to the rhizosphere. Ecology Letters 8*:* 1264-1270

Olsson P.A. *et al.* 2005. The influence of external nitrogen on carbon allocation to Glomus intraradices in monoxenic arbuscular mycorrhiza. New Phytologist 168: 677-686

Olsson P.A. *et al.* 2010 Carbon dynamics in mycorrhizal symbioses is linked to carbon costs and phosphorus benefits. FEMS Microbiology Ecology 72*:* 123-131

Omacini M. *et al.* 2012. Grass-endophyte symbiosis: A neglected aboveground interaction with multiple belowground consequences. Applied Soil Ecology 61: 273-279

Öpik M. *et al.* 2006. Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. Journal of Ecology 94: 778-790

Öpik M. *et al.* 2009. Large scale parallel 454 sequencing revealshost ecological group specificity of arbuscular mycorrhizal fungi in a bonomoral forest. New Phytologist 184: 424–437

Öpik M. *et al.* 2010. The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). New Phytologist 188: 223-241

Pace N.R. 2009. Mapping the Tree of Life: Progress and Prospects. Microbiology and Molecular Biology Review 73: 565-576

Pallara G *et al.* 2013. Transcript analysis of stress defence genes in a white poplar clone inoculated with the arbuscular mycorrhizal fungus *Glomus mosseae* and grown on a polluted soil. Plant Physiology & Biochemistry 63: 131-139

Palmer T.M. *et al.* 2010. Synergy of multiple partners, including freeloaders, increases host fitness in a multispecies mutualism. Proceedings of the National Academy Sciences of the United States of America 107: 17234-17239

Pardo F. *et al.* 1993. Isolation of verbascoside, an antimicrobial constituent of *Buddleja globosa* leaves. Journal of Ethnopharmacology 39: 221-222

Parniske M. 2004. Molecular genetics of the arbuscular mycorrhizal symbiosis. Current Opinion In Plant Biology 7: 414-421

Parniske M. 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nature Reviews, Microbiology 6: 763-775

Paszkowski U. 2006. Mutualism and parasitism: the yin and yang of plant symbioses. Current Opinion in Plant Biology 9:364-370

Pearson J.N. *et al.* 1993. Mediation of competition between two colonizing VA mycorrhizal fungi by the host plant. New Phytologist 123: 93-98

Pearson J.N. & Jakobsen I. 1993. Symbiotic exchange of carbon and phosphorus between cucumber and 3 arbuscular mycorrhizal fungi. New Phytologist 124: 481-488

Pellmyr O. & Huth C.J. 1994. Evolutionary stability of mutualism between yuccas and yucca moths. Nature 372: 257-260

Petersen H. & Luxton M. 1982. A comparative analysis of soil fauna populations and their role in decomposition processes. Oikos 39:288-388

Pfeffer P.E. *et al.* 1999. Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. Plant Physiology 120: 587-598

Pfeffer P.E. *et al.* 2004. The fungus does not transfer carbon to or between roots in an arbuscular mycorrhizal symbiosis. New Phytologist 163: 617-627

Phalan B. *et al.* 2011. Minimising the harm to biodiversity of producing more food globally. Food Policy 36: S62-S71

Phillips J.M. & Hayman D.S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society 55: 158-161

Posada D. & Crandall K.A. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817-818

Poulsen M. & Boomsma J.J. 2005. Mutualistic fungi control crop diversity in fungus-growing ants. Science 307: 741-744

Powell J.R. *et al.* 2009. Phylogenetic trait conservatism and the evolution of functional trade-offs in arbuscular mycorrhizal fungi. Proceedings of the Royal Society B-Biological Sciences 276: 4237-4245

Powell J.R. *et al.* 2011. Evolutionary criteria outperform operational approaches in producing ecologically relevant fungal species inventories. Molecular Ecology 20: 655-666

Pruesse E. *et al.* 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research 35: 7188-7196

Purvis A. & Hector A. 2000. Getting the measure of biodiversity. Nature 405: 212-219

Qiang X. *et al.* 2012. The mutualistic fungus *Piriformospora indica* colonizes *Arabidopsis* roots by inducing an endoplasmic reticulum stress-triggered caspase-dependent cell death. Plant Cell 24*:* 794-809

R Development Core Team. 2010. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/

Rasmussen N. *et al.* 2000. P-31 NMR for the study of P metabolism and translocation in arbuscular mycorrhizal fungi. Plant Soil 226: 245-253

Rasmussen L.S. *et al.* 2006. Transfer of iridoid glycosides from host plant *Galium verum* to hemi-parasitic *Euphrasia stricta*. Biochemical Systematics and Ecology 34: 763-765

Redecker D. *et al.* 2000. Glomalean fungi from the Ordovician. Science 289: 1920-1921

Reinhardt D. 2007. Programming good relations development of the arbuscular mycorrhizal symbiosis. Plant Biology 10: 98-105

Reinhart D. *et al.* 2012. Predicting plant responses to mycorrhizae: integrating evolutionary history and plant traits. Ecology Letters 15: 689-695

Requena N. *et al.* 2007. Plant signals and fungal perception during arbuscular mycorrhiza establishment. Phytochemistry 68:33-40

Rico-Gray V. 2001. Interspecific interactions. Encyclopedia of life sciences. Macmillan Publishers Ltd, Nature Publishing

Rillig M.C. 2004. Arbuscular mycorrhizae and terrestrial ecosystem processes. Ecology Letters 7: 740-754

Rillig M.C. & Mummey D.L. 2006. Mycorrhizas and soil structure. New Phytologist 171: 41-53

Rodriguez R.J. *et al.* 2008. Stress tolerance in plants via habitat-adapted symbiosis. International Society of Microbial Ecology Journal. 2: 404-416

Rodriguez R.J. *et al.* 2009. Fungal endophytes: diversity and functional roles. New Phytologist 182: 314-330

Rohlfs M. *et al.* 2007. Secondary chemicals protect mould from fungivory. Biology Letters 5: 523-525

Rohlfs M. & Churchill A.C.L. 2011. Fungal secondary metabolites as modulators of interactions with insects and other arthropods. Fungal Genetics and Biology 48: 23-34

Ronquist F. *et al.* 2011. MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice across a Large Model Space. Systematic Biology 61:539-542

Rowe H.I. *et al*. 2007. Comparisons of mycorrhizal responsiveness with field soil and

commercial inoculum for six native montane species and *Bromus tectorum*. Restoration Ecology 15:44–52

Roze V.L. *et al.* 2011. Compartmentalization and molecular traffic in secondary metabolism: A new understanding of established cellular processes. Fungal Genetics and Biology 48: 35-48

Ruyter-Spira C. *et al.* 2013. The biology of strigolactones. Trends in Plant Science 18:72-83

Ryan M.H. *et al.* 2005. Reduced growth of autumn-sown wheat in a low-P soil is associated with high colonisation by arbuscular mycorrhizal fungi. Plant Soil 270: 275-286

Sachs J.L. *et al*. 2004. The evolution of cooperation. The Quarterly Review of Biology 79:135-160

Salvioly P. & Bonfante A. 2013. Systems biology and "omics" tools: A cooperation for next-generation mycorrhizal studies. Plant Science 203:107-114

Sambrook J. *et al.* 1989. Molecular Cloning: A Laboratory Manual, 2nd edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY)

Sanders I.R. *et al.* 1995. Identification of ribosomal DNA polymorphisms among and within spores of the Glomales-Applications to study on the genetic diversity of Arbuscular Mycorrhizal fungal communities. New Phytologist 130: 419-427

Sanders I.R. and Croll D. 2010. Arbuscular mycorrhiza: the challenge to understand the genetics of the fungal partner. Annual Review of Genetic 44: 271-292

Santos J.C. *et al.* 2006. Molecular analysis of arbuscular mycorrhizal fungi colonising a semi-natural grassland along a fertilisation gradient. New Phytologist 172: 159-168

Santos-Gonzalez J.C. *et al.* 2007-Seasonal dynamics of arbuscular mycorrhizal fungal communities in roots in a seminatural grassland. Applied Environmentall Microbiology 73: 5613-5623

Schaarschmidt S. *et al.* 2007. Regulation of arbuscular mycorrhization by carbon. The symbiotic interaction cannot be improved by increased carbon availability accomplished by root-specifically enhanced invertase activity. Plant Physiology 143: 1827-1840

Schädler M. *et al.* 2005. Is palatability of a root-hemiparasitic plant influenced by its host? Oecologia 146: 227-233

Schenck N.C. & Smith G.S. 1985. 2 new dimorphic species in the endogonaceae-*Glomus-ambisporum* and *Glomus-heterosporum*. Mycologia 4: 566-574

Scheublin T.R. *et al.* 2004. Non Legumes, legumes, and root nodules harbor dif ferent arbuscular mycorrhizal fungal communities. Applied and Environmental Microbiology 70: 6240-6246

Schnoor T.K. *et al.* 2011. Mechanical soil distur-bance as a determinant of arbuscular mycorrhizal fungal communities in semi-natural grassland. Mycorrhiza 21: 211-220

Schoch C.L. *et al.* 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barecode marker for fungi. Proceedings of the National Academy of Sciences of the United States of America 109: 6241-6246

Schreiner R.P. & Bethlenfalvay G.J. 2003. Crop residue and Collembola interact to determine the growth of mycorrhizal pea plants. Biology and Fertility of Soils 39: 1-8

Schüßler A. *et al.* 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycological Research 105:1413-1421

Schüßler A. & Walker C. 2010. The Glomeromycota. A species list with new families and new genera. Gloucester, Published in libraries at The Royal Botanic Garden Edinburgh, The

Royal Botanic Garden Kew, Botanische Staatssammlung Munich, and Oregon State University; electronic version freely available online at www.amf-phylogeny.com

Schüßler A. *et al.* 2011. Revealing natural relationships among arbuscular mycorrhizal fungi: Culture Line BEG47 represents *Diversispora epigaea*, not *Glomus versiforme*. PLoS ONE 6: e23333

Schwartz M.W. & Hoeksema J.D. 1998. Specialization and resource trade: biological markets as a model of mutualisms. Ecology 79:1029-1038

Selosse M. A. & Le Tacon F. 1998. The land flora: a phototroph-fungus partnership? Trends in Ecology and Evolution 13:15-20

Selosse M.A. *et al.* 2006. Mycorrhizal networks: des liaisons dangereuses? Trends in Ecology & Evolution 21: 621-628

Sesterhenn K. *et al.* 2007. Occurrence of iridoid glycosides in in vitro cultures and intact plants of *Scrophularia nodosa* L. Plant Cell Reports 26: 365-371

Seufferheld M.J. & Curzi M.J. 2010. Recent discoveries on the roles of polyphosphates in plants. Plant molecular Biology Reporter 28: 549-559

Sheng M. *et al.* 2012. Growth of corn roots and associated arbuscular mycorrhizae are affected by long-term tillage and phosphorus fertilization. Agronomy Journal 104: 1672-1678

Sikes B.A. *et al.* 2010. Deciphering the relative contributions of multiple functions within plant-microbe symbioses. Ecology 91:1591-1597

Simmons D.R. *et al.* 2009. Lobulomycetales, a new order in the Chytridiomycota. Mycological Research 113: 450-460

Simms E.L. & Taylor D.L. 2002. Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. Integrative and Comparative Ecology 42: 369-380

Simon N. *et al.* 1992. Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Applied Environmental Microbiology 58: 291-295

Simon L. *et al.* 1993. origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363: 67-69

Simon N. *et al.* 1994. Characterization of oceanic photosynthetic picoeukaryotes by flow cytometry. Journal of Phycology 30:922-35

Šmilauerová M. & Šmilauer P. 2002. Morphological responses of plant roots to heterogeneity of soil resources. New Phytologist 154: 703-715

Smith S.E. *et al.* 1994a. Transfer of phosphate from fungus to plant in VA mycorrhizas: calculation of the area of symbiotic interface and of fluxes of P from two different fungi to *Allium porrum L*. New Phytologist 127: 93-

Smith S.E. *et al.* 1994b. Nutrient transport in mycorrhizas: structure, physiology and consequences for efficiency of the symbiosis. Plant & Soil 159: 103-113

Smith F.A. *et al.* 2000. Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago trunculata*. New Phytol 147: 357-366

Smith S.E. *et al.* 2003. Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. Plant Physiology 133: 16-20

Smith S. & Read D. 2008. Mycorrhizal Symbiosis, Edition 3. Academic Press and Elsevier London ISBN 978-0-12-370526-6

Smith F.A. *et al.* 2009. More than a carbon economy: nutrient trade and ecological sustainability in facultative arbuscular mycorrhizal symbioses. New Phytologist 182*:* 347-358

Smith S.E. *et al.* 2010. Plant performance in stressful environments, interpreting new and established knowledge of the roles of arbuscular mycorrhizas. Plant Soil 326: 3-20

Smith S.E. & Smith F.A. 2011. Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. Annual Review in Plant Biology 62: 227-250

Staaden S. *et al.* 2010. Fungal toxins affect the fitness and stable isotope fractionation of Collembola. Soil Biology & Biochemistry 42: 1766-1773

Stockinger H. *et al.* 2009. *'Glomus intraradices* DAOM197198', a model fungus in arbuscular mycorrhiza research, is not *Glomus intraradices*. New Phytologist 183: 1176-1187

Stout M.J. *et al.* 1996. Identity, spatial distribution, and variability of induced chemical responses in tomato plants. Entomologia Experimentalis et Applicata 79: 255-271

Strack D. *et al.* 2003. Arbuscular mycorrhiza: biological, chemical, and molecular aspects. Journal of Chemical Ecology 29: 1955-1979

Streitwolf-Engel R. *et al.*1997. Clonal growth traits of two Prunellaspecies are deter-mined by co-occurring arbuscular mycorrhizal fungi from a calcareous grassland. Journal of Ecology 85: 181-191

Taberlet P. *et al.* 1991. Universal primers for amplification of three noncoding regions of chloroplast DNA. Plant Molecular Biology 17:1105-1109

Takanishi I. *et al.* 2009. Short-chain polyphosphate in arbuscular mycorrhizal roots colonized by *Glomus spp*.: A possible phosphate pool for host plants. Soil Biology & Biochemistry 41: 1571-1573

Tanabe Y. *et al.* 2004. Molecular phylogeny of Zygomycota based on EF-1α and RBP1 sequences: limitations and utility of alternative markers to rDNA. Molecular Phylogenetics & Evolution 30: 438-449

Tanaka Y. & Yano K. 2005. Nitrogen delivery to maize via mycorrhizal hyphae depends on the form of N supplied. Plant Cell & Environment 28: 1247-1254

Tanaka A. *et al.* 2012. Fungal endophytes of grasses. Current Opinion in Plant Biology 15: 462-468

Taylor J.W. *et al.* 2000. Phylogenetic species recognition and species concept in fungi. Fungal Genetic & Biology 31: 21-32

Teutsch Hausmann N. & Hawkes C.V. 2010. Order of plant host establishment alters the composition of arbuscular mycorrhizal communities. Ecology 91:2333-2343

Thiéry O. *et al.* 2010. Evolutionary dynamics of introns and homing endonuclease ORFs in a region of the large subunit of the mitochondrial rRNA in Glomus species (arbuscular mycorrhizal fungi, Glomeromycota). Molecular Phylogenetics & Evolution 55: 599-610

Thonar C. *et al.* 2012. Real-time PCR to quantify composition of arbuscular mycorrhizal fungal communities, marker design, verification, calibration and field validation. Molecular Ecology Resource 12: 219-232

Tian C.J. *et al.* 2010. Regulation of the nitrogen transfer pathway in the arbuscular mycorrhizal symbiosis: Gene characterization and the coordination of expression with nitrogen flux. Plant Physiology 153: 1175-1187

Tilman D. & El Haddi A. 1992. Drought and biodiversity in grasslands. Oecologia 89:257–264

Tilman D. 1996a. Biodiversity: population versus ecosystem stability. Ecology 77:350–363

Tilman D. 1996b. Productivity and sustainability influenced by biodiversity in grassland ecosystems. Nature 379: 718-720

Tilman D. *et al.* 2001. Forecasting agriculturally driven global environmental change. Science 292:281-284

Trappe J. 1987. Phylogenetic and ecologic aspects of myco-trophy in the angiosperms from an evolutionary stand-point. In: Safir GR, ed. Ecophysiology of VA mycorrhizal plants. Boca Raton, Florida, USA: CRC Press. 5-25

Tulasne L.R. and Tulasne C. 1845. Fungi non nulli hipogaei, novi v. minus cognito act. Giornale Botanico Italiano 2: 55-63

Van Beneden P.J. 1875. Les commensaux et la parasites dans le reigne animal. Paris, Librairies Germer Baillères et C^{ie}

van der Heijden M.G.A. *et al.* 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396: 69-72

van der Heijden M.G.A. *et al.* 2003. Different arbuscular mycorrhizal fungi alter coexistence and resource distribution between co-occurring plants (vol 157, pg 569, 2003). New Phytologist 158: 601-601

van der Heijden M.G.A. *et al.* 2004. Arbuscular mycorrhizal fungi as support systems for seedling establishment in grassland. Ecology letters 7: 293-303

van der Heijden M.G.A. *et al.* 2006a. Symbiotic bacteria as a determinant of plant community structure and plant productivity in dune grassland. FEMS Microbiology Ecology 56: 178-187

van der Heijden M.G.A. *et al.* 2006b. The mycorrhizal contribution to plant productivity, plant nutrition and soil structure in experimental grassland. New Phytologist 172: 739-752

van der Heijden M.G.A. & Scheublin T.R. 2007. Functional traits in mycorrhizal ecology, their use for predicting the impact of arbuscular mycorrhizal fungal communities on plant growth and ecosystem functioning. New Phytologist 174: 244-250

van der Heijden M.G.A. *et al.* 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11: 296-310

Vandenkoornhuyse P. & Leyval C. 1998. SSU rDNA sequencing and PCR-fingerprinting reveal genetic variation within *Glomus mossae*. Mycologia 90: 791-797

Vandenkoornhuyse P. *et al.* 2001. High genetic diversity in arbuscular mycorrhizal fungi: evidence for recombination events. Heredity 87: 243-253

Vandenkoornhuyse P. *et al.* 2002a. Extensive fungal diversity in plant roots. Science 295: 2051

Vandenkoornhuyse P. *et al.* 2002b. Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. Molecular Ecology 11: 1555-1564

Vandenkoornhuyse P. *et al.* 2003. Co-existing grass species have distinctive arbuscular mycorrhizal communities. Molecular Ecology 12: 3085-3095

Vandenkoornhuyse P. *et al.* 2007. Active root-inhabiting microbes identified by rapid

incorporation of plant-derived carbon into RNA. Proceedings of the National Academy of Sciences of the United States of America 104: 16970-16975

Varga S. 2010. Review. Effects of arbuscular mycorrhizas on reproductive traits in sexually dimorphic plants. Spanish Journal of Agricultural Research 8: S11-S24

Vaz A.B.M. *et al.* 2012. Arbuscular mycorrhizal colonization of Sorghum vulgare in presence of root endophytic fungi of *Myrtus communis*. Applied Soil Ecology 61: 288-294

Verbruggen E. & Kiers E.T. 2010. Evolutionary ecology of mycorrhizal functional diversity in agricultural systems. Evolutionnary Applications 3:547-560

Verbruggen E. *et al.* 2010. Positive effects of organic farming on below-ground mutualists, large-scale comparison of mycorrhizal fungal communities in agricultural soils. New Phytologist 186: 968-979

Verbruggen E. *et al.* 2012. Spatial structure and inter-specific cooperation: Theory and an empirical test using the mycorrhizal mutualism. American Naturalist 179: E133-E146

Verheggen F.J. *et al.* 2012. Aphid alarm pheromone as a cue for ants to locate aphid partners. PLOS ONE 7: e41841

Vierheilig H. 2004. Regulatory mechanisms during the plant-arbuscular mycorrhizal fungus interaction. Canadian Journal of Botany 82:1166-1176

Vilgalys R. 2003. Taxonomic misidentification in public DNA databases. New Phytologist 160: 4-5

Vosatka *et al.* 2012. Development of arbuscular mycorrhizal biotechnology and industry: current achievements and bottlenecks. Symbiosis: 58: 29-37

Wagg C. *et al.* 2011a. Belowground biodiversity effects of plant symbionts support aboveground productivity. Ecology Letters 14: 1001-1009

Wagg C. *et al.* 2011b. Mycorrhizal fungal identity and diversity relaxes plant-plant competition. Ecology 92*:*1303-1313

Waller F. *et al.* 2005. The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance and higher yield. Proceedings of the National Academy of Sciences of the United States of America 102: 13386-13391

Wardle D. A. *et al.* 2003. Long term effects of wildfire on ecosystem properties across an island area gradient. Science 300: 972-975

Weiner J. *et al.* 2010. Evolutionary agroecology, the potential for cooperative, high density, weed-suppressing cereals. Evolutionary Applications 3:473-479

West S.A. & Herre E.A. 1994. The ecology of the new world fig-parasitizing wasps idarnes and implications for the evolution of the fig-pollinator mutualism. Proceeding of the Royal Society B 258: 67-72

White T.J. *et al.*1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ editors. PCR protocols, a guide to methods and applications. San Diego, California Academic Press pp. 315-322

Wilcke *et al.* 2000. Coarse woody debris in a montane forest in Ecuador: mass, C and nutrient stock, and turnover. Forest Ecology & Management 205:139-147

Wilson *et al.* 2012. Arbuscular mycorrhizal fungi improve the growth and nodulation of the annual legume messina (*Melilotus siculus*) under saline and non-saline conditions. Crop & Pasture Science. 63: 164-178

Woese C.R. 2000. Interpreting the universal phylogenetic tree. Proceedings of the National Academy of Sciences of the United States of America 97: 8392-8396

Wurst S. *et al.* 2004. Combined effects of earthworms and vesicular-arbuscular mycorrhizas on plant and aphid performance. New Phytologist 163: 169-176

Wurst S. *et al.* 2008. Intraspecific variation in plant defense alters effects of root herbivores on leaf chemistry and aboveground herbivore damage. Journal of Chemical Ecology 34: 1360-1367

Wurst S. *et al.* 2010. Microorganisms and nematodes increase levels of secondary metabolites in roots and root exudates of *Plantago lanceolata*. Plant and Soil 329: 117-126

Xing X. *et al.* 2012. Mutualism breakdown in breadfruit domestication. Proceedings of the Royal Society B 279: 1122–1130

Xu P. *et al.* 2007. Phylogenetic analysis of the sequences of rDNA internal transcribed spacer (ITS) of *Phytophthora sojae*. Journal of Genetic & Genomic 34: 180-188

Yue Q. *et al.* 2000. Isolation and characterization of fungal inhibitors from *Epichloe festucae*. Journal of Agricultural and Food Chemistry. 48: 4687-4692

Zak D.R. *et al.* 2003. Plant diversity, soil microbial communities, and ecosystem function: are there any links? Ecology 83:2042-2050

Zhu Y.G. *et al.* 2001. Phosphorus (P) efficiencies and mycorrhizal responsiveness of old and modern wheat cultivars. Plant Soil 237:249–255

Acknowledgments :

Réaliser un doctorat c'est toute une aventure, un peu comme des montagnes russes avec des hauts bien hauts et des bas très bas, des moments de stress et de travail acharné, alternés avec des moments plus détendus. On y apprend des choses passionnantes et on y fait aussi de très belles rencontres dont font partie la plupart des gens que je vais remercier ici. Je vais donc essayer de procéder du plus professionnel au plus personnel même si certaines des personnescitées transgressent ces frontières et vont donc se retrouver un peu partout !!

Tout d'abord, je voudrais remercier le ministère de l'éducation pour le financement de thèse dont j'ai pu bénéficier, l'UEB et l'Université de Rennes1 pour les bourses qui m'ont permis d'aller travailler un an à Amsterdam, et l'ANR ECS pour le financement des expériences.

Thank you to Marc-André Selosse, Matthias Rillig, Nancy Johnson and Nico van Straalen for accepting to judge my work and be part of my thesis committee.

Thank you to all the teams who welcomed me during this PhD: to Dave Tilman for his advices and to his team at the Cedar Creek LTER for all their help during the sampling campaigns, to Jan Jansa for my two stays in Swiss to Eschikon (ETH Zurich) and for his kindness and help during the qPCR analyses, thank you to Nico van Straalen and his team for welcoming me in the Animal Ecology laboratory in Amsterdam and everyone from there I knew for a year, Maria, Valentina, Daniel, Yummy, Martha, Eric, thank you for the help you gave me in setting up experiments, Oscar thank you for your help in DNA and RNA extractions, and during ultracentrifugations, Astra thank you for the work you have done on the experiment with fungivorous and secondary metabolites; Martje thank you for counting spores and fungi on the roots. And of course thank you to Cécile, the first French I met in Amsterdam after 10 months spent over there! Thank you for all the good moments! Et enfin, un grand merci à Ecobio et plus particulièrement à l'étage 2 du batiment 14b et aux deux expatriés du 14a : Amandine, Achim, Alexis, Philippe, Alexandrine, Nathalie, Frédérique, Myriam, André-Jean, Sarah, Steph et Flore pour les moments de détente, les bons petits gâteaux du vendredi lors des pauses café et des repas de midi, les verres en ville et les sorties badminton.

And now, I would like to thank Philippe and Toby, my two great supervisors! Thank you

for trusting me and giving me the opportunity to achieve this thesis which has been very rewarding and has given me the opportunity to travel to the United States, the Netherlands, the Switzerland, while working on the fascinating subject which is mutualism between plants and arbuscular mycorrhizal fungi. Thank you to both of you for always being present and available and for all the constructive comments you made on my work. Philippe, a big thank you for your unfailing optimism, your plan B and even C, D and E when the experiments do not work, your high availability even when you were in Minneapolis for these two very stressful weeks before returning the manuscript and of course, thank you for the pair of angora socks! Toby, thank you for your energy, your enthusiasm, your perfectionism, your answers and fast corrections despite the 800 miles that separate our two labs. Thanks to both of you for comforting me in my want to continue my career in research.

Merci à Sophie et Alexandra, qui ne se sont jamais départie de leur sourire et de leur bonne humeur en dépit de toutes les difficultés que mes échantillons ont pu leur apporter ! Merci les filles pour votre patience, votre gentillesse et pour le travail acharné que vous avez effectué sur ces fichus amplicons! Et en parlant de séquençage, merci à Achim et Alexis pour toute l'aide qu'ils ont apporté au niveau bioinformatique et au labo pour les tests d'amorces et le design des MID in silico.

Amandine et Dung, seulement un an et demi que l'on se connait et malgré tout déjà une belle amitié, plein d'excellents moments passés ensemble, de soirées inoubliables et de fous rires partagés ! Ma petite Amandine, question boulot, merci pour ton aide pour le choix des amorces, le design des MIDs (souviens-toi de tous ces ctrl C, ctrl V pour le calcul des ΔG!!) et pour m'avoir accompagnée lors de la campagne d'échantillonnage de mai dernier. Merci, bien sûr, pour le super séjour à New York qui a suivi! Se retrouver du mauvais côté de Central Park à 6h du matin après une nuit blanche alors qu'on ne rêve que d'une chose : aller dormir, ça n'a pas de prix !! Merci aussi pour toutes ces séances de sports, tous les potins échangés pendant nos footings, et pour tes encouragements lors de la période de rédaction! Ma petite Dung, la maman du trio et la reine des baguettes, merci à toi aussi pour ton calme, ta gentillesse, ces séances de sport qu'on a partagé toutes les trois, et pour toutes les merveilles culinaires que tu nous as concoctées: les nems, rouleaux de printemps et bien d'autres, et trăm phần trăm !!

Elo, ma stagiaire de rêve ! Merci pour ton aide dans mes manips et mes échantillonnages. Comme Amandine tu as souffert avec moi de ces séjours à Cedar Creek !! Décalage horaire, lever 6h, fin du boulot 20h, chaleur écrasante, clim hors-service et tornades ! Vive le Minnesota au printemps ! Merci aussi pour toutes les musiques et les clips hautement

intellectuels, raffinés et de bon gout que tu as pu m'envoyer pour me distraire au bureau « oh oh oh mon dieu mais que voisje ? C'est l'amourrrr, pour toujourrrrrs » ou encore « Choke me etc...» du grand poète Xzibit. Et merci pour ton aide précieuse lors de l'analyse du séquençage en urgence juste avant que je rende mon manuscrit.

Guillaume et Céline, merci pour les fêtes jusqu'au bout de la nuit, trous spatio-temporels, univers parallèles qui commencent à 20h et se terminent à 5h sans qu'on ne les voit passer, et pour les concerts, soirées et festivals électro ! Céline, un petit merci en plus pour mes réfs !!

Il est maintenant temps de remercier ma plus belle rencontre lors de ces trois années et demie de thèse, Merci mon Steph pour ton amour, tes encouragements, ton aide, la complicité que l'on a, pour tout ce qu'on partage au quotidien et dans le désordre: toutes les musiques bien pourries que tu as pu me mettre dans la tête, les séances de bad, les céréales et le lait bien frais du dimanche matin, ma page de garde de thèse, les publis à imprimer, ton humour et tes imitations. A quelques jours près, on est entré dans la période intense de la rédaction de thèse ensemble, et une grande partie des moments de stress s'est transformée en pétage de plombs collectif et grands fous rires, un grand merci pour ça aussi!

Un grand merci à mes parents et mes deux frangins pour m'avoir toujours supportée et encouragée, accompagnée et soutenue pendant tous les moments importants de ma vie, et pour m'avoir poussée à toujours essayer d'aller plus loin ! Merci pour ces retours de weekend à Cherbourg chouchoutée et le coffre de la voiture plein de bonnes petites choses à grignoter et pour vos visites à Amsterdam.

Merci à tous les doctorants avec qui j'ai partagé le bureau 216, j'ai eu beaucoup de chance, tous se sont avérés particulièrement sympathiques ! Merci à Nico pour son calme à toute épreuve, à Flore pour sa grande connaissance de R, et aux deux Steph. Un merci tout particulier à Steph ♀ pour toute l'aide que tu m'as apportée au labo de biologie moléculaire quand je suis arrivée à Ecobio en master 2.

Merci à Audrey et Libou, mes deux inséparables de la fac de Caen; à Lydie (la meilleure des cousines), à Seb, à Fifounette et Gégé pour leur amitié de toujours !

Merci aux deux géosciences Batman et Robin pour avoir fait partie du 'hard nucleus' des dernières sorties ! Quelques indices sur leur identité : « pétite à pétite » et scrotum de kangourou !

Et enfin merci à tous les autres pour les bons moments passés ensembles Maël, Benj,

Philippe L, Merwann, et j'en passe...!!

Ah ! Et j'oubliais ! Pour leur dévouement et leur sacrifice, je voudrais rendre un hommage à toutes les bestioles du bureau !! Merci aux phasmes pour avoir subi les paris diverses et variés, la famine et la sécheresse ; aux poissons morts au combat ; et aux collemboles d'Amsterdam aspirés soufflés, affamés, et enfin déséchés pour la science !

Résumé :

Evolution de la Coopération et Conséquences d'une Baisse de Diversité de Plantes sur la Diversité des Symbiontes Racinaires.

Doctorant: Marie Duhamel.
Directeurs de thèse: Philippe Vandenkoornhuyse et E. Toby Kiers
Tuteur: Didier Andrivon.

Introduction:

Les symbioses, des « interactions entre au moins deux différents organismes vivants en association physique étroite, généralement à l'avantage des deux », sont des relations très importantes au niveau du fonctionnement des ecosystèmes. Différentes règles permettent d'expliquer la stabilité de certaines symbioses. Ces méchanismes sont : la transmission vertical, le choix du partenaire et la purge génomique. Cependant une symbiose très importante au niveau des écosystemes terrestres ne respecte pas ces différentes règles: C'est la symbiose mycorhizienne à arbuscules.

Cette symbiose existant entre les plantes et les champignons mycorhiziens arbusculaires (AMFs) date d'environs 450 millions d'années et touche plus de 80% des plantes. Des échanges nutritifs ont lieu au niveau des arbuscules et des cellules racinaires. Des produits carbonés issus de la photosynthèse sont fournis par la plante aux champignons, et du phosphate, de l'eau et d'autres nutriments vont des AMFs vers les plantes. L'implication forte de ces deux organismes au niveau du cycle du carbone et du phosphore font de cette symbiose un élément clé du bon fonctionnement et de la productivité des écosystèmes. Un même champignon AM peut coloniser plusieurs plantes et une même plante peut être colonisée par plusieurs AMFs. Le maintien de ce mutualisme est devenu particulièrement important dans le contexte actuel de perte de biodiversité. Un des objectifs de cette thèse était de comprendre la stabilité de ce mutualisme. L'accent a tout d'abord été mis sur les échanges de nutriments impliqués dans cette symbiose, en testant si la plante hôte et les symbiotes fongiques sont capables de discriminer leurs différents partenaires, et d'allouer davantage de ressources aux partenaires fournissant plus de nutriments. J'ai ensuite étudié la possibilité de l'implication de la plante hôte dans la protection des symbiotes mycorhiziens via un transfert de métabolites secondaires dans les hyphes. Nous avons alors pu emettre une nouvelle hypothèse suggérant que la protection en métabolites secondaires venant de la plante serait positivement corrélée avec le niveau de coopération (à savoir le transfert des nutriments) du champignon symbiotique. L'echelle d'étude est ensuite passée de l'individu à la communauté en étudiant les effets de la diminution de la diversité végétale sur la diversité des symbiotes racinaires. Pour ce faire, des analyses moléculaires et des outils novateurs ont été utilisés, tels que le séquençage à haut débit. Pour faciliter encore l'étude des

séquences obtenues et d'autres séquences fongiques, j'ai collaboré avec des collègues afin de créer une base de données 'Phymyco-DB' rendue publique en 2012. Enfin, je discute de l'implication du mutualisme mycorhizien dans le contexte des systèmes agricoles actuels et propose de nouvelles trajectoires pour gérer ces systèmes.

Chapitre 1: Des récompenses réciproques stabilisent la coopération dans la symbiose mycorhizienne à arbuscules.

Dans ce contexte de co-mycorhization, certains champignons vont tendre à diminuer leurs apports pour augmenter leur fitness, ces champignons auraient dû être selectionnés et preferenciellement proliférer au cours de l'évolution, la coopération plantes-AMFs n'aurait alors pas été aussi répandue ni aussi ancienne. Comment s'est donc maintenue cette symbiose? Quelles sont les causes de sa stabilité?

Hypothèse: La plante-hôte controlerait le développement des champignons arbusculaires les moins coopératifs en limitant les apports de carbone vers ces champignons.

Deux expériences indépendantes de impliquant un marquage à l'isotope stable du carbone le 13C (ou 'Stable Isotope Probing') ont été mises en place avec des plants de *Medicago truncatula* inoculés avec 1,2 ou 3 AMFs variant dans leur niveau de coopération. Les *M. truncatula* ont été placés dans une chambre de marquage afin d'être enrichis en 13C. Les ARNs des racines ont ensuite été extraits, puis utracentrifugés dans du césium trifluoroacétate. Un gradient de densité se forme. En fonction de la quantité de carbone transféré de la plante vers le champignon, l'ARN de celui-ci sera plus ou moins dense et sera donc à une place différente le long du gradient. Les ARNs sont fractionnés, puis une transcription inverse et une PCR quantitative sont effectuées pour chaque fraction.
Le but est de rechercher l'ARN le plus enrichi en 13C donc celui qui apparait dans les fractions les plus denses, et de voir si l'ARN du champignon le plus coopératif est présent dans ces fractions.
Après une analyse GLM, il a été démontré qu'en effet l'ARN du champignons arbusculaire le plus coopératif est significativement plus dense, il reçoit donc plus de carbone provenant de la plante. La stabilité de la relation coopérative entre les plantes et les AMFs au cours du temps peut donc être expliquée par le fait que la plante peut détecter les champignons et leur niveau de coopération et controler le développement des AMFs les moins coopératifs par une limitation de l'apport de carbone. Cela va ainsi favoriser les symbiontes les plus coopératifs.

Parallèlement, des experiences *in vitro* mettant en jeu des boites de petri divisées en trois

compartiments, un colonisé par des racines et les deux autres colonisés par des champignons arbusculaires coopératifs et non-coopératifs, ont été mises en place afin de confirmer les résultats précédement cités. Ces expériences *in vitro* ont permi de manipuler le niveau de phosphore transmis par les champignons AM (quel que soit leur niveau de coopération) aux plantes et le niveau de carbone transmis pas les plantes aux champignons AM. Les résultats obtenus ont confirmés les résultats de l'experience de SIP pour le comportement des plantes. De plus, ils ont aussi mis en évidence la stratégie de rétention que les champignons AM les moins coopératifs adoptent, incorporant le phosphate qu'ils acquièrent dans des chaines polyphosphatées non assimilables par les plantes plutot que de leur transmettre se phosphate directement comme le font les champignons coopératifs.
La stabilité de cette symbiose mycorhizienne à arbuscule semble donc être expliquée par le contrôle bilatéral effectué par les deux partenaires. Chacun d'entre eux récompensant l'autre à la hauteur de ce qu'il a fourni.

Chapitre 2: La présence de fongivores déclenche t-elle le transfert de métabolites de protection provenant des plantes hôtes vers les hyphes des champignons mycorhiziens à arbuscules?

Comme cela a été vu dans l'experience précédente (chapitre 1), la stabilité de la symbiose AM s'effectue par un controle bilateral par les deux organismes mis en jeu via des échanges nutritifs plus ou moins important en fonction de leur niveau de coopération. Ce contrôle pourrait aussi s'effectuer via l'implication des plantes-hôtes dans la protection de leur symbiontes AM. Les métabolites secondaires sont des composés chimiques communément synthétisés par les plantes et impliqués dans leur défense contre diverses menaces. Il est connu que la colonisation des racines par les champignons arbusculaires mycorhiziens induit des changement dans la concentration de métabolites secondaires contenus dans les racines ou les feuilles des plantes hôtes. Considérant également que différents composés provenant de la plantes peuvent être transférés aux champignons MA, nous emettons l'hypothèse suivante:

Hypothèse: La plante serait impliquée dans la défense des champignons arbusculaires mycorhiziens colonisant ses racines via un transfert de métabolites secondaire vers les hyphes.

Une expérience a été mise en place avec des plants de *Plantago lanceolata* colonisés par *G.intraradices* (le plus coopératif) et *G. custos* (le moins coopératif) (seuls ou en mélange) en présence ou en absence de *F. candida,* un insecte fongivore. Les analyses prévues étaient une

mesure de la biomasse des *P. lanceolata*, de la biomasse des hyphes, un comptage des *F. candida*, une mesure des taux de trois métabolites secondaires (verbascoside, catalpol et aucubine) dans les feuilles, les racines et les hyphes par HPLC pour tous les traitements.
Cependant, *G. custos* n'a pas réussi à se développer, rendant impossible ces analyses dans les différents traitements contenant ce champignon MA. Un autre problême est aussi apparu au niveau du comptage des *F. candida*, il est donc impossible de savoir si elles se développent mieux en présence de *G. intraradices*. Mais il a quand même pu être démontré que *Glomus intraradices* a un effet positif sur la croissance de *P. lanceolata* en présence ou non de *F. candida* et que les *F. candida* consomment bien des hyphes de *G.intraradices*. De plus l'expérience a montré la présence de catalpol dans les hyphes de *G.intraradice*s uniquement dans les traitements contenant *F. candida*. Ce métabolite secondaire serait donc potentiellement transféré de la plante au champignons MA, en présence de *F. candida*, la plante pourrait donc protéger cet AMF des insectes fongivores en lui transférant ces subtances protectrices. Ce résultat amène donc une nouvelle hypothèse quant au contrôle et à la stabiité de la symbiose MA:

Hypothèse: La plante transfèrerait moins de métabolites secondaires aux champignons les moins coopératifs, ceux ci seraient donc moins protégés et seraient préférenciellement consommés par les insectes fongivores.

Chapitre 3: PHYMYCO-DB: Une base de données 'curée' pour les analyses de la diversité et de l'évolution fongique.

Contexte:
Dans les études de séquençage à partir d'échantillons environnementaux, les champignons peuvent être identifiés sur la base des séquences d'acides nucléiques, en utilisant soit des séquences très variables utilisées en tant que codes barres permettant l'identification d'espèces, ou des séquences conservées contenant un signal phylogénétique de haute qualité. Pour ces derniers, l'identification repose sur des analyses phylogénétiques et l'adoption du concept d'espèce phylogénétique.
Une telle analyse exige que les séquences de référence soient bien identifiées et déposées dans des bases de données d'accès public. Cependant, de nombreuses entrées dans ces bases de données de séquences publiques sont problématiques en termes de qualité et de fiabilité et de ce fait, l'utilisation de ces données requiert une vérification préalable pour assurer une interprétation phylogénétique correcte.

Méthodes et les conclusions principales:

Pour faciliter les inférences et affectations phylogénétique, nous introduisons une base de données de séquence fongique. La base de données PHYMYCO-DB comprend des séquences fongiques issues de GenBank qui ont été filtrées pour satisfaire à des critères de qualité des séquences strictes. Pour la première version, deux marqueurs taxonomiques moléculaires largement utilisés ont été choisis: la petite sous unité de l'ARNr nucléaire (ssurRNA) et des séquences du gènes EF1-α. Suite une extraction automatique et une filtration des séquences, une curation manuelle est effectuée pour éliminer les séquences problématiques tout en préservant des séquences pertinentes utiles pour les études phylogénétiques. À la suite de curation, ~ 20% des séquences automatiquement filtrées ont été retirées de la base de données. Pour démontrer comment PHYMYCO-DB peut être utilisée, nous avons testé un ensemble de séquences de champignons Chytridiomycètes environnementaux obtenus à partir d'échantillons prélevés eneaux profondes.

Conclusion:
PHYMYCO-DB offre les outils nécessaires pour: (i) extraire des séquences fongiques de haute qualité pour chacun des 5 phylums fongiques, à tous les niveaux taxonomiques, (ii) extraire des alignements déjà effectués, pouvant être utilisés comme «alignements de référence» , (iii) lancer des alignements de séquences personnelles associées à des données stockées. Un total de 9120 ssuARNr et 672 séquences fongiques EF1-α de haute qualité sont maintenant disponibles. La création de cette base de donnée à permis de faciliter les analyses des séquences obtenues dans les expériences du chapitre 4.

Chapitre 4: Conséquences d'une baisse de diversité de plantes sur la baisse de diversité des symbiontes racinaires (résultats préliminaires):

Les microorganismes du sol et les symbioses racinaires sont très importants pour la nutrition des plantes, et il a été démontré qu'une plus grande diversité de plantes mène à une plus grande productivité (Tilman *et al.*, Nature 1996, 2001), de plus on sait qu'il y a une corrélation positive entre la productivité de l'écosystème et la diversité des champignons mycorhiziens (Van der Heijden *et al.*, Nature 1998). Considérant ces liens entre diversité et productivité au sein d'un écosystème ainsi que l'existance d'une préférence d'hôte entre plante et champignons MA, une hypothèse peut alors être émise:

Hypothèse: Une baisse de diversité des plantes conduirait à une érosion de la diversité des symbiontes racinaires. Une perte importante des microorganismes du sol aurait un impact négatif sur la fertilité du sol. Cela pourrait être une des conséquences des monocultures intensives actuelles. Répondre à cette hypothèse pourrait mener à une nouvelle gestion des

sols prenant en compte les bénéfices de ces mutualismes.

Cinq campagnes d'échantillonnage ont été effectuée au sein du LTER de Cedar Creek dans le Minnesota (US). Ce dispositif, développé par Dave Tilman en 1994, est composé de différentes parcelles comportant une diversité variable de plantes (i.e. 1, 2, 4, 8 et 16 espèces par parcelle). Ces plantes appartiennent aux quatre groupes fonctionnels forbs, légumineuses, C3 et C4. Ces campagnes ont eu lieu entre Juin 2011 et Mai 2013. Des racines, du sol et des feuilles des plantes présentes ont été récoltés. A partir de ce matériel, une extraction des acides nucléiques a été effectuée, suivie par une amplification de la petite sous unité ribosomique (ssurRNA) ciblant les champignons MA, et enfin par un pyroséquencage (Next Generation Sequencing GSFLX 454 de Roche) afin d'obtenir la diversité de ces symbiontes racinaires.
Pour l'analyse phylogénétique des séquences obtenues, la base de donnée Phymyco-DB contenant des séquences 18S et ef1alpha des 5 phyla de champignons a été utilisée ainsi qu'un pipeline d'analyse permettant un tri et une assignation des séquences obtenue de manière rapide et efficace. Jusqu'à maintenant seul un run de pyroséquençage contenant des échantillons provenant de parcelles avec 1 à 8 espèces de plantes a pu être analysé. Les résultats présentés ici sont donc préliminaires. Ils permettent cependant de faire ressortir plusieures observations: le nombre d'OTUs observés dans cette étude ainsi que les l'assignation taxonomique des taxa qu'ils contiennent reflètent ce qui a déjà été observé dans d'autres études sur la diversité des champignons mycorhiziens arbusculaires, avec une majorité des taxa observés issue de la famille des Glomérales. La diversité de ces champignons semble diminuer lorsque la diversité des plantes diminue confirmant ainsi notre hypothèse. La structuration de la communauté de champignons arbusculaires change aussi en fonction de la diversité de plantes présentes. Certains OTUs (unité taxonomique opérationnelle) sont équitablement représentés dans tous les types de parcelles alors que d'autres sont uniquement observés en présence d'une faible diversité de plantes. A l'inverse, d'autres OTUs sont majoritairement présents dans les parcelles contenant 4 et 8 espèces de plantes. Il y a donc un remaniement de la communauté fongique lié au changement de diversité de plantes.
D'autre analyses vont suivre et venir compléter ces résultats préliminaires. En effet, la diversité des champignons de manière plus globale ainsi que la diversité des bactéries associés aux racines vont maintenant être étudiées. En considérant les cinq campagnes, les dynamiques de ces communautés aux cours des saisons et des années vont pouvoir être déterminées. Cela permettra aussi l'aquisition de données sur le fonctionnement de ces communautés, et les synergies et compétitions qui peuvent potentiellement avoir lieu au sein de ces organismes.

Chapitre 5: L'agriculture mondiale durable: de nouvelles trajectoires possibles à partir des symbioses mutualistes.

Nous sommes actuellement confrontés un problème majeur consistant à répondre aux demandes en nourriture de la population mondiale. Parrallèlement à cette demande alimentaire croissante due à l'essor démographique, la productivité agricole fait face à un ralentissement en raison du réchauffement climatique, de la dégradation de l'environnement ainsi que de l'épuisement des stocks de phosphore. L'équation est très simple: la production alimentaire doit être assez élevée tout en minimisant les dommages à l'environnement. La productivité, mais pas la taille, des zones agricoles déjà existantes doit être améliorée. La biodiversité doit être préservée avec par exemple la sélection des plantes sur la base de la sélection de groupe. Ces solutions sont intéressantes, mais ne considèrent pas le compartiment souterrain et plus particulièrement les symbiontes racinaires que sont les champignons arbusculaires mycorrhiziens. Ces organismes pourraient être des acteurs clés pour inverser la tendance. En raison des multiples services qu'ils fournissent, ils ont le potentiel d'améliorer la santé des plantes et la productivité des sols. Cependant les pratiques mises en oeuvre dans l'agriculture conventionnelle représentent une menace majeure de la symbiose à champignons mycorhiziens arbusculaires. Ces pratiques diminuent la diversité fongique, l'efficacité et la stabilité de la symbiose. C'est un cercle vicieux car, à son tour, la diminution de l'efficacité des symbiontes arbusculaires affecte négativement les plantes et la productivité des systèmes agricoles. Sur la base de récentes découvertes sur le contrôle de la symbiose AM, de nouvelles trajectoires dans les pratiques de gestion des sols agricoles et l'amélioration des performances des plantes qui prennent en compte le compartiment du sous-sol et l'évolution de la stratégie mutualiste, sont proposées ici. Dans ce contexte, nous soutenons que les selectionneurs ont la possibilité d'utiliser les symbiose mycorhizienne à arbuscules endémiques pour une agriculture innovante et écologiquement intensive. Dans cet article, des hypothèses sur l'implication de symbiose MA ainsi que de nouvelles pratiques de gestion sont proposées. A travers l'adoption de mesures simples, comme la diminution ou l'arret des labours, le maintien d'une certaine diversité de plantes, une selection des cultivars plus adaptées ainsi que la prise en compte d'autre mutualismes, la promotion de la symbiose MA pourrait être une étape majeure de la mise en place d'une agriculture durable.

Discussion et Perspectives:

L'objectif de cette thèse était d'aborder les aspects essentiels régissant la symbiose mycorhizienne arbusculaire. Pour atteindre cet objectif, j'ai effectué une série d'analyses et d'expériences allant du niveau individuel de la plante-hôte au niveau des communautés végétales. Nous avons examiné les processus de discrimination, en se demandant si à la fois les plantes et les champignons MA peuvent détecter le niveau de transfert des éléments nutritifs provenant de l'autre partenaire. Nous avons ensuite étudié potentiel effet protecteur que peuvent avoir une plante hôte envers ses symbiotes fongiques AM. Nous avons également utilisé

des méthodes moléculaires innovantes pour étudier les effets d'une variation de la richesse en espèces des plantes-hôtes sur la diversité des communautés fongiques AM. Cependant d'autres expériences sont à envisager afin d'approfondir notre connaissance sur le symbiose AM et de mieux comprendre son implication dans le fonctionnement des ecosystèmes.

L'expérience de SIP devra être réitérée en mettant en jeu des champignons AM provenant d'autres familles, en effet tous les champignons MA n'ont pas les mêmes stratégies de colonisations ce qui pourrait avoir des conséquences sur leur efficacité en tant que symbionte.

Toujours en dans le cadre d'une expérience de SIP, des champignons MA et/ou des plantes provenant de sol agricoles gérés de manières conventionnels devraient être utilisés afin de confirmer si oui ou non les plantes issues de ces sols ont perdu leur trait de sanction et si les champignons MA selectionnés par ces pratiques sont moins coopératifs.

Les conséquences des associations entre certaines espèces plantes et certaines espèces de champignons MA ainsi que l'hypothèse de l'utilisation par la plante de ressources en excèes pour entretenir la symbiose doivent être explorées de manières plus approfondie. Les mecanismes moléculaires cachés derrière le trait de sanction de la plante devront être mis à jour via des études de transcriptomique.

L'hypothèse d'une implication de la plante dans la protection de ses symbiontes AM proportionnelle au niveau de coopération de ces symbiontes devra aussi être vérifiée.

Les différentes approches afin d'étudier les interactions plantes-microorganismes sont généralement réductionnistes en raison de la complexité de ces relations. Peu à peu, nous devons maintenant augmenter le niveau de complexité de nos expériences en étudiant plusieurs symbiotes en interaction avec une seule plante-hôte, puis au sein de plusieurs hôtes afin d'obtenir une vision plus complète de la symbiose MA et de ses interactions au sein d'un écosystème

Ce projet de thèse apporte un nouvel éclairage sur la façon dont fonctionnent ces interactions entre les plantes et champignons MA et sur la manière dont ils façonnent les processus écologiques et les trajectoires évolutives dans les écosystèmes naturels et agricoles. Ces points sont d'une importance majeure pour développer une agriculture plus écologiquement intensive et durable. Le projet a fourni de nouvelles connaissances et perspectives sur la perte de la diversité végétale, et ses conséquences pour la stabilité de la symbiose AM. Comme les champignons mycorhiziens sont essentiels dans les processus des écosystèmes et l'entretien de la fertilité des sols, ce travail devrait avoir un large impact dans (i) la politique de protection des sols, (ii) la recherche sur l'amélioration des plantes et (iii) la conception de systèmes agricoles durables.

Mots Clés: mutualisme, plante, champignons mycorhiziens à arbuscules, diversité, communautés, coopération, fonctionnement des écosystèmes, allocation de carbone, métabolites secondaires. SSU rRNA, séquençage de masse.