



Doctoral Thesis

Synthetic mycorrhizal communities Establishment and functioning

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**SYNTHETIC MYCORRHIZAL COMMUNITIES –
ESTABLISHMENT AND FUNCTIONING**

A dissertation submitted to

ETH ZURICH

for the degree of

Doctor of Sciences

presented by

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List of abbreviations

AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
BEG	La Banque Européenne des Glomales
DW	Dry weight
HLD	Hyphal length density
LSD	Least significant difference
LSU	Large ribosomal subunit
NM	Non-mycorrhizal
P	Phosphorus
<i>p</i>	Probability level
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RLC	Root length colonization
RMSE	Root mean squared error
SA	Specific activity

Summary

Communities of arbuscular mycorrhizal fungi (AMF) were established in roots of *Medicago truncatula* under controlled conditions, using fungal isolates, which have previously been obtained from a single field site. Their composition was dissected using novel real-time PCR approach, which allowed species-specific quantification of the different AMF both in roots and in the soil. Together with the traditional staining-microscopy approach, these tools have been used to describe the ontogeny of root colonization of medic plants by five different AMF species. Significant diversity in root colonization patterns among the different species was described, along with the magnitude and temporal trajectories of phosphorus (P) acquisition benefits provided by the fungi to the plant. This diversity of plant responses was further characterized in a compartmented system coupled with dual radioisotope labeling (^{32}P and ^{33}P) of a root-free zone. This allowed assessment of contribution of the morphological and physiological traits of the external mycorrhizal mycelium to the P acquisition by the plants and comparison of the mycorrhizal carbon (C) costs associated with the established symbiosis. More precisely, *Gigaspora margarita* was collecting P only from a limited soil volume, establishing dense mycelium networks close to the roots. Following P uptake by the fungus from the soil, the P was immobilized in the fungal mycelium before being delivered to the plant. The extent of soil exploration by two different *Glomus* species was higher than that by *Gigaspora margarita*. They both provided differential amounts of P to the plants. However, since the less efficient P-gatherer *Glomus claroideum* also required significantly less C from the plants than *G. intraradices*, the levels of growth promotion by both of the *Glomus* species were similar. Competitive abilities of these three AMF species were then assessed in all possible two-species combinations using a range of inoculum densities of the involved fungal species. The results indicated limited competition and frequent facilitation between members of the AMF community. Facilitation was particularly strong if phylogenetically distant fungal species were combined. Functional complementarity with respect to P uptake and plant growth was observed in some AMF combinations sharing the same plant root system.

Zusammenfassung

Gemeinschaften arbuskulärer Mykorrhizapilze (AMF) wurden unter kontrollierten Bedingungen in *Medicago trunculata* Wurzeln etabliert. Dabei wurden Pilzisolat verwendet, die von derselben Ackerfläche stammten. Die Zusammensetzung der Mykorrhizagemeinschaften wurde durch Verwendung eines neuartigen real-time PCR Ansatzes aufgegliedert, so dass die verschiedenen AMF sowohl in Wurzeln als auch im Boden artspezifisch quantifiziert werden konnten. Diese Werkzeuge wurden zusammen mit der traditionellen mikroskopischen Färbemethode verwendet, um die Ontogenese der Wurzelkolonisierung bei *Medicago* durch fünf verschiedene AMF-Arten zu beschreiben. Bedeutende Unterschiede im Kolonisierungsschema der einzelnen Arten wurden beschrieben, ebenso wie Ausmass und der zeitlicher Verlauf des Zugewinns bei der Phosphor-(P)-Aufnahme, den die Pilze der Pflanze verschaffen. Diese Diversität in der Wirkung auf die Pflanze wurde weiterhin in einem Kammersystem durch doppelte Radioisotopenmarkierung (^{32}P und ^{33}P) der wurzelfreien Kammer charakterisiert. Dadurch konnten die Beiträge von morphologischen und physiologischen Eigenschaften des externen Myzels zur pflanzlichen P-Aufnahme erhoben und der mit der Symbiose verbundene Kohlenstoff-(C)-Aufwand verglichen werden. Im Detail nahm *Gigaspora margarita* durch dichte, wurzelnahe Myzelgeflechte nur aus einem begrenzten Bodenvolumen P auf. Dieses wurde nach der Aufnahme aus dem Boden im Pilzmyzel festgesetzt, bevor es der Pflanze zugeführt werden konnte. Die Flächenausnutzung durch zwei unterschiedliche *Glomus*-Arten war höher als durch *Gigaspora*. Beide lieferten den Pflanzen unterschiedliche Mengen P. Da der weniger leistungsfähige P-Beschaffer *Glomus claroideum* aber signifikant weniger C von den Pflanzen benötigte als *G. intraradices*, war die Wachstumsförderung durch beide *Glomus*-Arten ähnlich. Die Wettbewerbsfähigkeit dieser drei AMF-Arten wurde dann in allen möglichen Zwei-Arten-Kombinationen erhoben, wobei die Inokulumdichten der beteiligten Pilzarten variiert wurden. Die Ergebnisse deuteten auf begrenzte Konkurrenz und häufig auf eine gegenseitige Förderung zwischen Mitgliedern der Pilzgemeinschaft hin. Förderung war besonders stark ausgeprägt, wenn phylogenetisch entfernte Pilzarten kombiniert wurden. Funktionelle Ergänzung im Bezug auf P-Aufnahme und Pflanzenwachstum wurde bei einigen AMF-Kombinationen beobachtet, die dasselbe Wurzelsystem teilten.

Résumé

Des communautés de champignons mycorhiziens à arbuscules (AMF) ont été établies en conditions contrôlées dans des racines de *Medicago truncatula*, en utilisant des isolats fongiques préalablement isolés d'un même champ. Leur composition a été disséquée en utilisant une nouvelle approche basée sur la PCR en temps réel qui rend possible la quantification spécifique au niveau des espèces de différents AMF lorsque présents dans les racines et le sol. Combinés à l'approche traditionnelle reposant sur la coloration et la microscopie, ces outils ont été employés pour décrire l'ontogénèse de la colonisation de racines par cinq espèces différentes d'AMF. Une diversité significative a été observée parmi ces différentes espèces dans le profil de colonisation des racines ainsi que dans l'amplitude des bénéfices résultant de la symbiose concernant l'acquisition de phosphore (P) et du temps nécessaire pour leur apparition. Cette diversité de réponses de plantes fut ensuite caractérisée dans un système compartimenté couplé à un double marquage radioisotopique (^{32}P and ^{33}P) d'une zone non accessible aux racines. Cela permit l'évaluation de la contribution des traits morphologiques et physiologiques du mycélium mycorhizen extra-racinaire à l'acquisition de P par la plante ainsi que la comparaison des coûts mycorhiziens en carbone (C) associés à l'établissement de la symbiose. Plus précisément, il fut montré que *Gigaspora* prélevait du P uniquement dans un volume limité de sol, y établissant un réseau dense de mycélium à proximité des racines. Suite au prélèvement de P du sol par le champignon, le P fut immobilisé dans le mycélium fongique avant d'être livré à la plante. L'étendue de l'exploration du sol par deux espèces différentes de *Glomus* fut supérieure à celle par *Gigaspora*. Ils fournirent tous les deux des quantités différentes de P aux plantes. Cependant, puisque *Glomus claroideum*, montré pour être moins efficace à prélever le P, requière en même temps significativement moins de C des plantes que *Glomus intraradices*, les niveaux de promotion de la biomasse par les deux espèces de *Glomus* étaient similaires. Les capacités compétitives de ces trois espèces d'AMF furent ensuite évaluées dans toutes les combinaisons possibles de deux espèces, en utilisant une gamme de densités d'inoculum des espèces fongiques impliquées. Les résultats indiquèrent l'occurrence de peu de compétitions mais de fréquentes facilitations entre les membres de la communauté des AMF. Les facilitations étaient particulièrement marquées si les espèces fongiques

combinées étaient phylogénétiquement distantes. Des phénomènes de complémentarité fonctionnelle ont été observés à l'égard de l'acquisition de P et de la croissance de la plante dans certaines combinaisons d'AMF partageant le même système racinaire.

General introduction

Arbuscular mycorrhizal fungi

Arbuscular mycorrhiza (AM) is a widespread symbiosis between a monophyletic group of arbuscular mycorrhizal fungi (AMF) and plants (Smith & Read, 2008). The fungal partners of this symbiosis have recently been reclassified on the basis of DNA sequences into a separate phylum of fungi, the Glomeromycota (Schussler *et al.*, 2001). They are very ancient organisms, possibly dating back to Ordovician time, more than 450 million years ago (Redecker *et al.*, 2000). Through their roles in nutrient uptake, AM fungi were probably involved in the colonization of land by plants (Redecker *et al.*, 2000; Heckman *et al.*, 2001). Nowadays, AM fungi are found in nearly all terrestrial ecosystems, from tropical forests through prairies to the cold shores of Antarctica where they colonize the roots of the majority of land plant species. So far, only 150 to 200 species of AMF are recognized worldwide (Morton & Bentivenga, 1994; Redecker & Raab, 2006), establishing symbiosis with some 200 000 plant species. This results in a high degree of unspecificity among partners, although preferential associations between fungal and plant species have been recognized (Bever, 2002; Jansa *et al.*, 2002; Sanders, 2003). This limited specificity has as a consequence that one root system of a plant can be potentially colonized by different AM fungi (Van Tuinen *et al.*, 1998). The composition and diversity of AMF communities colonizing roots and soil have been recognized to be influenced by the plant species, the soil properties and the ecosystem management (Boddington & Dodd, 2000; Egerton-Warburton & Allen, 2000; Jansa *et al.*, 2002; Jansa *et al.*, 2006; Mathimaran *et al.*, 2007; Pivato *et al.*, 2007). Those and other studies indicated that usually more than 10 AMF species belonging to several genera were present in a single ecosystem and that, as said before, up to 5 AMF species were coexisting within the same root fragment (Daft & Hogarth, 1983; Van Tuinen *et al.*, 1998; Jansa *et al.*, 2003b).

Role of AMF in plant nutrition and biomass production

Upon colonization of plant roots, AMF penetrate the rhizodermis and occupy root cortex with hyphae growing through intercellular spaces. They establish intimate contact with living cellular protoplasts by extensive branching and/or coiling of their hyphae, forming typical structures known as arbuscules and hyphal coils (Dickson *et al.*, 2007; Smith & Read, 2008). Afterwards, the fungi start producing extraradical mycelium that will grow out of the colonized root and link the structures within the roots directly to the soil particles. These connections are functioning as extensions of the root system for uptake of mineral nutrients from the soil (Dodd *et al.*, 2000). Establishment of symbiosis is usually followed by an improved uptake of nutrients essential to plants (phosphorus, zinc, nitrogen and copper), especially with mycorrhiza-responsive plant species and/or in low fertility soils. This improved uptake usually results in an improved plant biomass production that can reach up to 2000% compared to the equivalent non-mycorrhizal plants (Powell, 1975; Smith & Read, 2008). In fertile soils and/or with unresponsive plant species such as maize, tomato or wheat, the growth of plants can be unaffected or lower upon colonization with AMF (Smith *et al.*, 2003; Smith *et al.*, 2004; Jansa *et al.*, 2005). In exchange to mineral nutrients, the plants deliver reduced carbon to the fungus, which, as a consequence, enhances the sink demand for carbon of the plant roots (Douds *et al.*, 1988; Jakobsen & Rosendahl, 1990; Lerat *et al.*, 2003). All in all, a great range of responses of plants to AMF has been described, ranging from positive to negative (Klironomos, 2003; Tawarayama *et al.*, 2003). This continuum of responses depends on the particular fungus-plant combination and on environmental conditions.

Functional diversity and complementarity

The formation and function of arbuscular mycorrhizas can be quite variable between fungal species and even between isolates of the same species (Munkvold *et al.*, 2004; van der Heijden *et al.*, 2004; Cavagnaro *et al.*, 2005; Hart & Reader, 2005; Jansa *et al.*, 2005). Functional diversity in AM refers to the differences observed between fungi in how they help host plants to resist biotic and abiotic stresses, although it is often understood in terms of plant growth responses only (Burleigh *et al.*, 2002; van der Heijden & Scheublin, 2007). The variation of plant responses has been observed and measured in

experiments, in which plants have mostly been inoculated with single AMF isolates under controlled conditions. This analysis of functional diversity with single AM species is certainly not fully relevant for field situations, where it is known that a single root system can be colonized by several AMF species. Under controlled conditions, only few studies have evaluated host plant performances (in terms of phosphorus (P) acquisition and biomass production) when plants were co-inoculated by several AMF species at the same time (van der Heijden *et al.*, 1998a; van der Heijden *et al.*, 2003; Gustafson & Casper, 2006; Jansa *et al.*, 2008). Usually, these studies have focused on the plant responses with quantifying only rarely the root occupancy of the inoculated AMF – and if they did, the results were not AMF species-specific and only semi-quantitative. This situation was mainly caused by the fact that methods traditionally used to detect AMF in roots were not fully quantitative due to methodical constraints, and also they did not allow discrimination of the different species. This lack of quantitative and specific tools has also limited the characterization of interactions occurring between different AMF species when competing for colonization of the same root system. As a consequence, the impact of simultaneous colonization of plant roots by functionally different AMF has been rarely analyzed (Lekberg *et al.*, 2007; Maherali & Klironomos, 2007; Jansa *et al.*, 2008). However, recent introduction of quantitative PCR (Alkan *et al.*, 2004; Isayenkov *et al.*, 2004) allowing quantitative assessment of their proper root occupancy is now providing strong tool to address these questions (Jansa *et al.*, 2008).

The theory of functional complementarity suggests that if a plant is colonized by several AMF species that show complementary functions (e.g. uptake of P from different soil pools), the host performance will be better than if the plant is colonized by any of the AMF species separately (Koide, 2000; Johnson *et al.*, 2004; Gustafson & Casper, 2006; Jansa *et al.*, 2008).

Objectives and approaches

To our opinion, the general approach used to provide strong evidences of functional complementarity should meet different requirements that were not always fulfilled in the past. Thus, the general objective of this PhD project was to create a framework, where these requirements could be met in order to provide new basis for better understanding of

functional complementarity and functional diversity of AMF communities, and to address competitive interactions between members of the community.

The analysis of functional complementarity requires:

1. The availability of inocula of many AMF species, preferentially isolated from the same ecosystem, and showing similar extent of root colonization at the time of harvest when administered singly.
2. The availability of detection tools allowing the species-specific quantification of several AMF species in roots after having been inoculated in mixture.
3. That AMF used in co-inoculation treatments are tested separately for their functions in regards to their ability to improve the P uptake and the plant biomass production.
4. That nature of interactions occurring between different AMF species when colonizing the same root system is assessed.
5. Working under controlled conditions to avoid the misinterpretations of results due to interactions with environmental conditions.

To meet these requirements, different experiments were carried out following the establishment of a model system and the development of AMF species-specific detection and quantification toolbox.

In chapter 1, the ability of different AMF species to colonize the roots was assessed in order to predict the conditions under which their inocula would provide a similar rate of root colonization after a certain time. This assessment was done for AMF species isolated from the same field in Switzerland. The root samples generated in this experiment were used to study the ontogeny of root colonization and to validate the detection tools developed in parallel. For the latter, the objective was to develop independent toolboxes based on molecular and immunological detections, respectively.

In chapter 2, the functions (in terms of P acquisition, plant C utilization, and biomass production) of the studied AMF were properly characterized when inoculated singly, and the diversity of plant responses analyzed with regards to the morphological and physiological traits of AMF.

In chapter 3, the nature of interactions occurring between the different AMF was addressed using the molecular tools, and the consequences for the plant of multiple colonizations analyzed in an additive design.

One of the prerequisites of the activities described in the chapters 2 and 3 was that plants inoculated by the studied AMF would present at harvest root colonization extent in the same range to prevent situations where plant responses and competitive abilities of AMF would be compared while plant roots would be colonized to completely different extents. Therefore, based on results obtained in the chapter 1 and in other experiments, experimental conditions and inoculum densities were adapted in order to achieve a minimum degree of RLC of 50% after 6 weeks of growth of *Medicago truncatula* (chapter 2 and 3).

Also, for practical reasons, from chapter 2 the continuation of the thesis was carried out without the AMF species *Glomus mosseae* (which did not grow well in the inoculum pots) and *Scutellospora pellucida* (which developed too slowly for achieving reasonable colonization levels in subsequent short-term studies).

These experiments were carried out in growth chambers, where the environmental conditions (temperature, light regime and humidity) were kept under precise control. The model plant was *Medicago truncatula* (cv. Jemalong J5) whose seeds were produced under controlled conditions in the greenhouse. The potting substrate was always the same and was consisting of a mixture of sands and soil from the field, where the AMF were originally isolated from. Details concerning the experimental set-up are provided in the concerned chapters. The review of literature concerning the specific topics is also presented in each of the chapters.

Chapter 1: Dynamics of *Medicago truncatula* root colonization by different arbuscular mycorrhizal fungi

Abstract:

Arbuscular mycorrhizal fungi (AMF) are ancient symbionts playing important roles in ecological processes. When establishing the symbiosis, AMF species demonstrate a differential ability of root colonization. This is usually referred to as infectivity. Dynamics of root colonization is usually studied by the combined staining-microscopy approach. Nevertheless, this method does not allow discrimination of different AMF species occupying the root and it is not fully quantitative. Here, we performed a large experiment on the dynamics of root colonization of *Medicago truncatula* by different AMF species, with the objective to describe the relationship between inoculum amount and time on the extents of root colonization. This was done to allow predictions of the colonization extents of the roots by different AMF species at different time points. This knowledge should allow establishment of plants colonized to similar extents by different AMF in subsequent functional experiments. We also established a molecular assay, based on a species-specific quantification of ribosomal DNA, to follow the dynamics of medic root colonization by five AMF species so as to allow quantification of development of each fungal species independently. These results showed a discrepancy between DNA quantification and staining-microscopy approach as measures of fungal biomass for fast colonizers such as *Glomus intraradices* and *Glomus claroideum*. The magnitude and trajectories of the dynamics in growth and phosphorus uptake benefits were different among the different AMF species, and this was likely related to the differences in colonization development and delivery of phosphorus to the plants by the different AMF species.

Keywords: *Glomus*; *Gigaspora*; large ribosomal subunit (LSU) copies; *Medicago truncatula*; model; ontogeny; root colonization; *Scutellospora*; time course, phosphorus, symbiotic benefits

Introduction:

Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with the majority of land plant species. While colonizing their root systems and the surrounding soil, they usually provide benefits to the plants in terms of nutrient acquisition, growth and tolerance to environmental stresses (Smith & Read, 2008). In addition, AMF play important roles in ecosystem functioning and are influencing inter- and intraspecific plant competitions and soil structure (Read & Perez-Moreno, 2003). It is now well established that AMF demonstrate an important functional diversity concerning the nutrient acquisition (Jakobsen *et al.*, 1992a; Jansa *et al.*, 2005). This functional diversity has been observed among AMF isolates belonging to different species, as well as among isolates of the same species (Munkvold *et al.*, 2004; van der Heijden *et al.*, 2004; Cavagnaro *et al.*, 2005; Hart & Reader, 2005). In natural and agricultural ecosystems, plant roots have been shown to be usually colonized by more than one AM species (Van Tuinen *et al.*, 1998), but the meaning of this multiple root occupancy for plant functioning is not yet well understood. The theory of functional complementarity proposed by Koide (2000) suggests that plants colonized by several AMF of complementary functions (e.g. uptake of nutrients from different soil pools) will receive more benefits than plants colonized by single AMF species. To fully appreciate this phenomenon, plants responses (improved plant biomass and phosphorus acquisition as compared to the non-mycorrhizal plants) upon colonization of the roots by different AMF species should be analyzed with regards to the mycorrhizal composition of the colonized roots. This has not been the case in the past, when experiments were carried out with multispecies inoculations of plants, addressing the functional complementarity within AMF communities. These experiments suffered from the lack of specific tools to quantify the occupancy of the roots by each of the inoculated AMF species/isolates. This roots occupancy results mainly from interaction events (see chapter 3) occurring between the different AMF species when colonizing and competing for the same root system. Therefore, more information has to be provided to understand competition outcomes between AMF species in order to link them to functioning of the AMF communities in the roots.

To do so, reliable tools must be developed to enable species-specific quantification of AMF development in the roots. In addition, root colonization by the studied AMF species

when single inoculated should be characterized prior to any competition tests because parameter such as infectivity of individual species will directly influence outcomes of mycorrhizal competition for root occupancy.

The process of root colonization (sometimes referred to as root infection) can be divided into a series of steps as follows: propagule germination, growth of the hyphae attracted by root exudates, root interception and formation of a primary entry point, ramification of mycelium within the root to form an infection unit, formation of secondary entry points and infection units (secondary spread), formation of extraradical hyphae, formation of propagules such as spores, and so on (Wilson, 1984a). The initiation of root colonization is mediated by exchange of signaling compounds between the two partners of the symbiosis (Akiyama *et al.*, 2005; Drissner *et al.*, 2007) and involves an important modification of their expressed genes (Brechenmacher *et al.*, 2004; Massoumou *et al.*, 2007).

In a loose sense, infectivity can be expressed as the amount, usually length, of root colonized by a given inoculum of a particular fungus within a defined time frame. If comparisons of infectivity are to be made between fungi, it is thus necessary to specify the environment, the host plant, the time frame and the inoculum density (Wilson & Tommerup, 1992). Progress of root colonization as expressed by fraction of root length colonized usually demonstrates an S-curve with a lag phase, an exponential increase and a saturation phase, where the maximal infection is achieved (plateau). The primary infection (penetration) and the secondary spread are important factors in the colonization of a root system and it is now commonly accepted that differential infectivities of AMF species are mainly due to their different modes of infection spread. Two strategies of root colonization have been recognized: the ability to spread within the root cortex to form large infection units, or the ability to form new infection units from “runner hyphae” creating secondary entry points.

The properties of the inoculum (inoculum density, activation stage of the propagules, and inoculum distribution around the roots) will also influence the root colonization, and so will do the environmental factors (among others: soil temperature, pH and available phosphorus status) (Wilson & Tommerup, 1992). As said before, proper characterization of colonization ontogeny appears important for competition studies and should be carried

out under controlled conditions to avoid interactions with the mentioned inoculum-related and environmental factors. Until now, few studies have been able to compare root colonization ontogeny of different AMF while standardizing these conditions (Hart & Reader, 2002c; Munkvold *et al.*, 2004; Hart & Reader, 2005). The other problem in describing root colonization is the fact that different methods are being used for assessing growth of fungal development in roots. The most widespread approach rely on visualization by staining of fungal structures (Phillips & Hayman, 1970; Vierheilig *et al.*, 2005) that can be combined with microscopy-based gridline intersection assessment to rank the colonization rates (McGonigle *et al.*, 1990). Nevertheless, it has been recognized that this method is not exactly quantitative and does not reflect the fungal biomass, mainly because it does not take into account the colonization intensity. Moreover, this method prevents the simultaneous detection and quantification of multiple AMF isolates within the root. Other methods have been proposed as alternatives to the microscopy-based approach and include the quantification of biomarkers such as fatty acids (Olsson *et al.*, 1995), chitin (Bethlenfalvay *et al.*, 1981; Toth *et al.*, 1991) or isoenzymes (Tisserant *et al.*, 1993), and more recently quantitative PCR (qPCR) (Alkan *et al.*, 2004; Isayenkov *et al.*, 2004; Jansa *et al.*, 2008). In spite of its accuracy and rapidity, this method has been criticized and some discrepancies were observed comparing this assessment of root colonization with the microscopic-based method (Jansa *et al.*, 2008). Nevertheless, this method is promising and is the only one, so far, allowing the quantitative detection of several AMF species co-colonizing the same root system.

Several objectives were followed in this work. First, it aimed at describing the dynamics of the roots colonization and of the P uptake benefits to *Medicago truncatula* inoculated by different AMF species, and at proposing regression models describing how the fraction of root length colonized (RLC) as assessed by staining-microscopy, and the plant benefits derived from the symbiosis, change in time. Based on that, the second objective was to predict the levels of root colonization by the different AMF species depending on their inoculum density and the age of plants. The reason for obtaining this knowledge was the need to establish comparable colonization levels by different AMF species for a given plant age in further competition studies, i.e. avoiding situations where one species would dominate the community simply because its inoculum potential would favor its

development and handicap development of another species. A third objective was to establish a quantitative assay for root colonization by different AMF species, allowing distinguishing different AMF species sharing the same root system. A model plant *Medicago truncatula* and optimized growth conditions were employed to allow strict reproducibility of the results.

Material and Methods:

Experimental setup

The experiment was carried out in plastic pots (700 ml) filled with a substrate that had been homogeneously mixed with the mycorrhizal inoculum. The substrate consisted of sterilized field soil, sterilized coarse quartz sand (grain diameter 0.7-1.2 mm) and sterilized fine quartz sand (grain diameter 0.08-0.2 mm) mixed in the ratio 1:3:1 (v:v:v). The soil was collected in Tänikon, Switzerland, air-dried, passed through 5 mm sieve and γ -irradiated at LEONI Studer Hard, Däniken, Switzerland, applying a dose of 25-75 kGy with ^{60}Co source. The available P content of the substrate was $21.9 \pm 0.43 \text{ mg kg}^{-1}$ (ammonium acetate-EDTA extraction, 1:10 w:v, 16h), and the readily available P pool ($E_{1\text{min}}$) was $1.73 \pm 0.06 \text{ mg kg}^{-1}$ as assessed by the isotope exchange kinetics approach (Frossard & Sinaj, 1997). Substrate C and N contents were 2.2 ± 0.1 and $0.24 \pm 0.01 \text{ g kg}^{-1}$, respectively.

Experimental design

One host plant and five AMF species were considered. Non-mycorrhizal treatment was included in the design as well. Forty pots were established for each of the 6 mycorrhizal treatments yielding 240 pots in total. For each mycorrhizal treatment, 5 inoculum doses were considered and were corresponding to pots containing 1, 5, 15, 40 and 100 g kg^{-1} of the fresh AMF inoculum, respectively, mixed with the whole substrate volume. These 30 treatments were replicated 8 times. One plant per treatment was harvested in weekly intervals during the 8 weeks after sowing.

Plants and AMF

Seeds of *Medicago truncatula* Gaertn. (medic) were surface sterilized for 10 min in concentrated (97%) sulphuric acid (Massoumou *et al.*, 2007) and then washed with sterile water and germinated for 3 days on moistened filter paper. Three seedlings were planted into each pot at the start of the experiment.

Five AMF species, all isolated from a single field in Switzerland (Jansa *et al.*, 2002) (Jansa, J. *et al.*, 2002) were used in this study. These were: *Glomus intraradices* BEG 158, *Glomus claroideum* BEG 155, *Glomus mosseae* BEG 161, *Gigaspora margarita* BEG 152 and *Scutellospora pellucida* BEG 153. The AMF inocula were produced in 1-kg pots and consisted of the same substrate as described above, planted with leek. After 12 months of growth, the inoculum pots contained high AMF spore and mycelium densities (Table 1). Non-mycorrhizal inoculum was produced under the same conditions as the inocula, and was checked for the absence of mycorrhizal propagules. It was supposed to contain the same bacterial and saprophytic fungal communities as the other AMF inocula.

The growing plants were watered every day and received 100 ml pot⁻¹ week⁻¹ of a modified Hoagland nutrient solution (Hoagland & Arnon, 1950) containing no P throughout the duration of the experiment. The pots were completely randomized in a growth chamber (Conviron PGV36, Winnipeg, Canada) under the following conditions: temperature 22/19°C and relative aerial humidity 75/90% (day/night, respectively), photoperiod 16 h, combined fluorescent and incandescent light, 330 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Harvest and measurements

Plants were harvested once a week for 8 weeks after sowing. Upon harvest, plant shoots were cut and dried at 70 °C for 72 h and weighed. Roots were washed from the substrate under tap water and then rinsed with deionized water, weighed and cut to about 1-cm pieces and mixed. Subsamples were taken for dry matter assessment and for root staining. On selected samples, additional root subsamples were taken for DNA extraction. The roots staining procedure was following the protocol described by Phillips and Hayman (1970) and Brundrett *et al.* (1984). Briefly, roots were macerated in 10% KOH (100g l⁻¹) at 90°C for 25 min, rinsed with water, incubated in 1% HCl (10 g l⁻¹) at room temperature for 1 h, and briefly rinsed with water before the transfer to 0.05% Trypan blue (0.5 g l⁻¹) in lactic acid: glycerol: water (1:1:1; v:v:v). In this solution, the roots were stained at 90°C for 2 h and then at room temperature overnight. Finally, the roots were de-stained for at least 24 h in water at room temperature. The extent of root length colonized by hyphae, arbuscules and vesicles was determined on stained roots according to the method of McGonigle *et al.* (1990), recording 50 root intersections per sample.

Table 1: List of AMF isolates and properties of their inocula used in this study

Species identity	Abbreviation	BEG number *	Spore density (g-1 dry weight)	Mycelium length density (m g ⁻¹ dry weight)	Proportion of dry to fresh mass (%)
<i>Glomus intraradices</i> Schenck & Smith	G. int	158	449	19.4	69
<i>Glomus claroideum</i> Schenck & Smith	G. clar	155	200	28.4	71
<i>Glomus mosseae</i> Gerd. & Trappe	G. moss	161	20	39.3	70
<i>Gigaspora margarita</i> Becker & Hall	Gi. m	152	135	27.3	68
<i>Scutellospora pellucida</i> Walker & Sanders	Scut. p	153	19	14.9	70

*Accession number in the International Bank for the Glomeromycota (www.kent.ac.uk/bio/beg/)

Dried shoot biomass samples were milled in a ball mill and incinerated at 550°C for 3 h, the ashes dissolved in 2 ml HNO₃ (65%), made to 50 ml with distilled water and filtered through 0.8-µm nitrocellulose filters. The concentration of phosphorus in the extracts was determined by Inductively-Coupled-Plasma Mass-Spectrometry (ICP-MS, Agilent 7500c, Agilent Technologies AG, Basel, Switzerland).

Quantification of root colonization by real-time PCR

PCR primers and hydrolysis (TaqMan) probes (Table 2) were designed for specific amplification of large ribosomal subunit (LSU) genes of the five different AMF species. The design of the primers and probes was carried out in AlleleID version 4 software (Premier Biosoft International, Palo Alto, California, USA) after having aligned (1) published LSU sequences of the respective fungal species from the public database and (2) cloned and sequenced fragments of DNA amplified from the AMF isolates used in this study. Non targeted AMF species were also included in the alignment in order to avoid cross-reactivity of the primers/probes with undesired species thus increasing the specificity of the molecular assay. More than 30 primer pairs were designed, synthesized and PAGE-purified at Microsynth (Balgach, Switzerland), tested for cross-reactivity using the five target AMF species, and the most suitable primers were further complemented by TaqMan probes (labeled with fluorescein and BHQ-1 quencher). The specificity of each assay (primers and probe) was cross-checked using genomic DNA extracted from spores of 26 different AMF isolates belonging to 16 different species, with no cross-species reactivity observed (data not shown). The real-time PCR quantification protocol for LSU copy numbers was established following the outline presented before (Jansa *et al.*, 2008). Briefly, DNA was extracted from single spores of the five target AMF species (Table 1) and further used as templates for PCR with LR1 and LR2 primers, using cycling conditions identical to those described previously (Jansa, J *et al.*, 2002). The concentration of LSU copy numbers (NC , copies l⁻¹) in each of these PCR products was calculated by knowing the amplicon length, L (761, 759, 767, 702 and 700 bp for *Glomus intraradices*, *Glomus claroideum*, *Glomus mosseae*, *Gigaspora margarita*, and *Scutellospora pellucida*, respectively), the DNA concentration of the sample (K , g l⁻¹,

determined by UV spectrophotometry) and molecular weight of DNA (660 Da bp⁻¹) using the following equation where, N_a is Avogadro's constant (6.023×10^{23}).

$$NC = \frac{K \times Na}{660 \times L} \quad (\text{Equation 1})$$

Table 2: Sequences of primers and TaqMan probes used for the real-time PCR quantification of large ribosomal subunit gene copies of the different AMF species and of the internal standard. The parameters **a** and **b** were used for conversion of the real-time PCR results into concentrations of gene copies per unit of volume (see Equation 3 in the Material and Methods section).

Target AMF species	Sequences (1): primer forward (5'→3') (2): primer reverse (5'→3') (3): TaqMan probe (5'→3')	Amplicon size (base pairs)	Annealing temperature (°C)	a	b
<i>Glomus intraradices</i>	TTCGGGTAATCAGCCTTTTCG (1) TCAGAGATCAGACAGGTAGCC (2) TTAACCAACCACACGGGCAAGTACA (3)	250	52	3.39	38.414
<i>Glomus claroideum</i>	GCGAGTGAAGAGGGAAGAG (1) TTGAAAGCGTATCGTAGATGAAC (2) AACAGGACATCATAGAGGGTGACAATCCC (3)	177	52	3.31	38.747
<i>Glomus mosseae</i>	GGAAACGATTGAAGTCAGTCATACCAA (1) CGAAAAAGTACACCAAGAGATCCCAAT (2) AGAGTTTCAAAGCCTTCGGATTTCGC (3)	122	54	3.53	42.299
<i>Gigaspora margarita</i>	CTTTGAAAAGAGAGTTAAATAG (1) GTCCATAACCCAACACC (2) TAACCTGCCAAACGAAGAAGTGC (3)	272	48	3.47	41.223
<i>Scutellospora pellucida</i>	AGAAACGTTTTTTACGTTCCGGGTTG (1) CCAAACAACCTCGACTCTTAGAAATCG (2) CCGTGTATACCAACCACTGGAATGTTATT (3)	127	54	3.41	41.614
Internal standard	CGAACCTGGACTGTTATGATG (1) AATAACAATCCCCTGTATTTTAC (2) CACCAGGCACCAACAACGACCATT (3)	87	50	3.33	40.581

Then, the PCR products were serially diluted to obtain billions to thousands of copies μL^{-1} . In addition to the AMF DNA preparations, we have used a pUC19 plasmid carrying a cassava mosaic virus DNA fragment (GenBank accession AJ427910) to be used as an internal standard to estimate the efficiency of DNA extraction from root samples. The above samples were used for optimizing and calibration of the real-time PCR assays, employing a LightCycler 2.0 (Roche Diagnostics, Rotkreuz, Switzerland) and Roche chemistry (LightCycler TaqMan Master). The cycling conditions were the following: initial denaturation at 95°C for 15 min, then 45 cycles with denaturation at 95°C for 10 sec, annealing at variable temperatures (Table 2) for 30 sec, and elongation at 72 °C for 1 sec. The cross-points (Cp) were recorded automatically by the LightCycler software and were used for establishment of calibration curves (see Calculation and Statistics section). Once the cycling conditions for the real-time PCR were established, the molecular assays were used to estimate the copy numbers of the fungal LSU in the roots. To do so, the DNA was extracted by DNeasy Plant Mini kit (Qiagen, Hombrechtikon, Switzerland) from the lyophilized samples (100-150 mg fresh roots) after homogenization by bead disruption twice for 15 s at high speed using a Mini-BeadBeater-8 (BioSpec Products, Bartlesville OK, USA) following the manufacturer's instructions. Before homogenization, all samples were spiked with a known number of the internal standard (5 billion copies). Quantification of the internal standard recovery in the DNA extracts, using real-time PCR with a specific primers pair and a TaqMan probe, allowed for a correction for both DNA losses during the extraction and presence of unspecific PCR inhibitors in the DNA extracts (Jansa *et al.*, 2008). All the real-time PCR reactions were performed in the LightCycler 2.0 using Roche chemistry with the specific primers and probes as described before, using the root DNA extracts diluted 5 times as templates, and total reaction volume of 9 μL (4.52 μL H₂O + 0.18 μL each primer (25 μM) + 0.07 μL TaqMan probe (25 μM) + 1.8 μL Roche Master Mix TaqMan + 2.25 μL template (root DNA extract diluted 5 \times)).

Calculation and Statistics

The levels of the root length colonized by the AMF (RLC; %) are given as the ratio of root intersections containing any of the mycorrhizal structures to all observed root intersections per sample, $\times 100$. These experimental data were used for establishment of

double-sigmoid regression models using the surface-fitting-website (<http://zunzun.com>). The equation was:

$$\text{RLC (\%)} \text{ estimated} = \frac{a}{\left(1 + e^{(b-c*\text{dose})}\right) * \left(1 + e^{(d-e*\text{time})}\right)} \quad (\text{Equation 2})$$

The number of LSU copies per unit of DNA extract volume was calculated by converting the Cp values from the real-time PCR amplification using the following equation:

$$\text{LSU copies (raw)} = 10^{((b-Cp)/a)} \times (\text{EV} \times \text{DF}) \quad (\text{Equation 3})$$

The values for the parameters **a** and **b** are listed in Table 2. EV refers to the final DNA extract volume (we have used 50 µl) and the DF to the dilution factor of the DNA extract, i.e. 5. The same equation was also used to calculate the number of copies of the internal standard recovered in the final DNA extract (i.e. Internal standard copies measured).

Subsequently, the concentrations of the LSU copies of the AMF species per unit of root dry mass were corrected for DNA losses/PCR inhibition as follows:

$$\text{LSU copies (corrected)} = \frac{\text{LSU copies (raw)}}{\text{Root dry weight (mg)}} \times \frac{\text{Internal standard copies spiked}}{\text{Internal standard copies measured}} \quad (\text{Equation 4})$$

Phosphorus concentrations in shoot extracts were used for calculation of the shoot P content. Shoot P uptake benefits (%) were calculated according to Cavagnaro *et al.* (2003), using individual shoot P content (mg) values of inoculated plants (Mp) and means of the shoot P content values of the non-mycorrhizal plants (NMP, means of five non-mycorrhizal treatment, pooling the 5 non-mycorrhizal inoculum doses treatments for every of the time points):

$$\text{P uptake benefit (\%)} = \frac{Mp - NMP}{NMP} \times 100 \quad (\text{Equation 5})$$

Results:

Extent of root length colonization by the five AMF assessed by microscopy:

At harvest, the extent of root length colonization (RLC) was assessed by traditional microscopy. For each AMF species, a total of 40 values were obtained and used in an attempt to model the RLC as a function of the inoculum dose and time of harvest. The regression models are shown in Figure 1a. The results revealed different patterns of RLC

response to the harvest time and inoculum dose between the different AMF species. For the three *Glomus* species, the amount of inoculum had virtually no influence on the RLC at any harvest time. The rates of root colonization for these AMF species appeared to be influenced solely by the age of the plants. For *Gigaspora margarita* and *Scutellospora pellucida*, the dose was importantly influencing the RLC, particularly for the low inoculum doses ($<15 \text{ g kg}^{-1}$).

In general, the most influential factor on the RLC levels was the time of harvest (Fig. 1). The shape of the response curve and the levels of colonization plateau (maximum RLC values) depended on the identity of the AMF species (Fig. 1a). *Glomus mosseae*, *Gigaspora margarita* and *Scutellospora pellucida* showed a sigmoid developmental trajectory, with markedly different lengths of the initial lag phase, whereas the developmental trajectory of the two other *Glomus* species was rather power-type without a prominent initial lag phase (Fig. 1). The levels of colonization plateau (maximum RLC values reached toward the end of the experiment) appeared to be determined by the identity of the AMF. The RLC values reached up to 100% for *Glomus intraradices*, 60% for *Glomus claroideum* and *Glomus mosseae* and 85% for *Gigaspora margarita*. The development of colonization by *Scutellospora pellucida* was still in its exponential phase towards the end of the experiment (Fig. 1). Establishment of the regression models for root colonization allowed for drawing of the contour plots, predicting the inoculum amounts to be applied in order to reach certain RLC level at a given time point (Fig. 1c). Except for *Scutellospora pellucida*, the contour plots are showing the calculated relationships for 25%, 50% and 75% RLC. It shows for instance that the medic roots will be colonized at 50% by *Glomus intraradices* at 4 weeks after inoculation, irrespective of the initial inoculum dose, or that *Scutellospora pellucida* needs 5 weeks before it does establish 10% RLC for the highest applied inoculum dose. The results indicated that there is only a very limited possibility to manipulate the RLC levels by changing inoculum dose for the *Glomus* species, whereas there is a potential to achieve different RLC levels for the *Gigaspora* and *Scutellospora* when manipulating their inoculum densities. For *Scutellospora* in particular, the levels of RLC on a short time are constrained by very long initial lag phase of the colonization trajectory.

Figure 1 (for legend see next page)

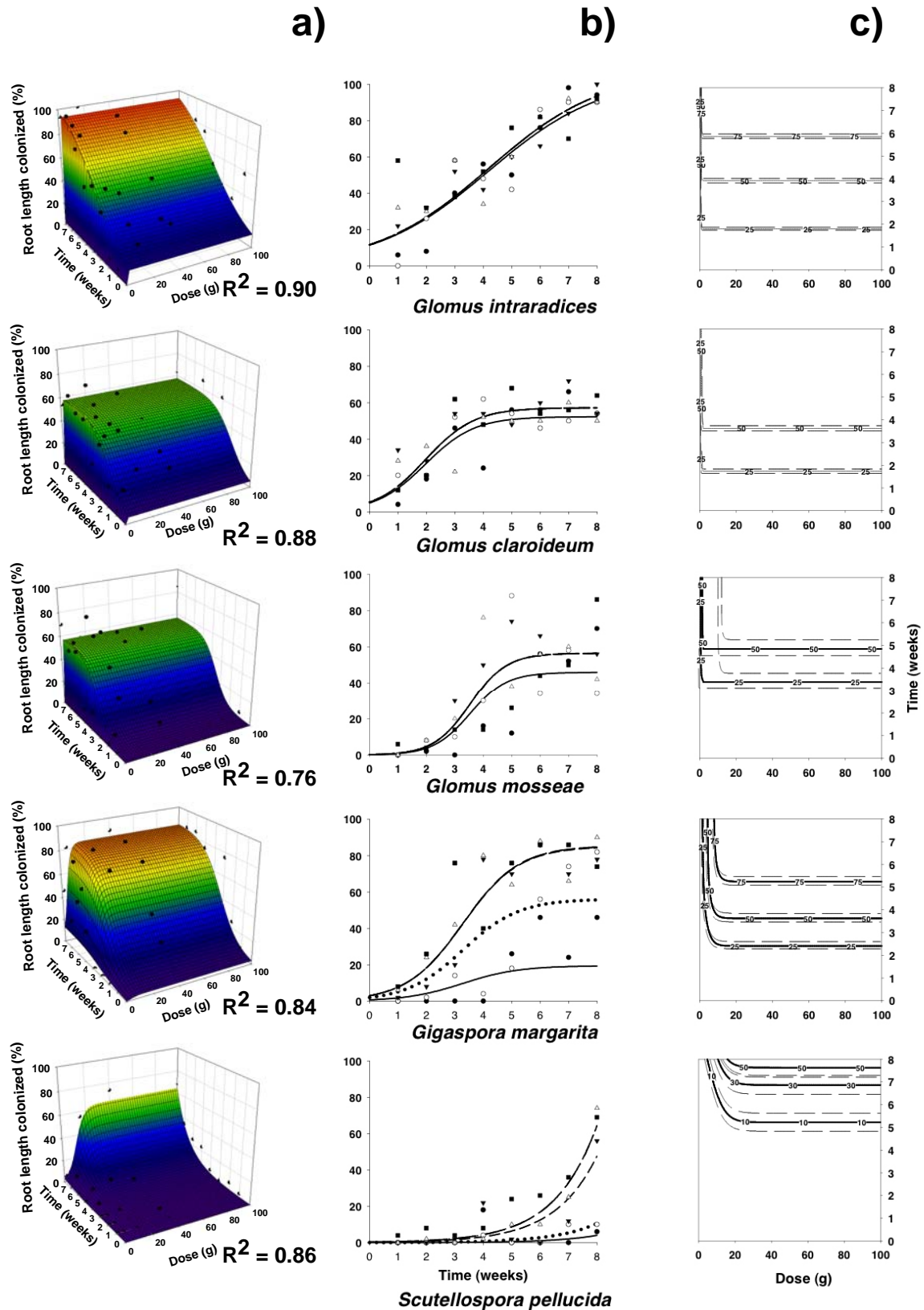
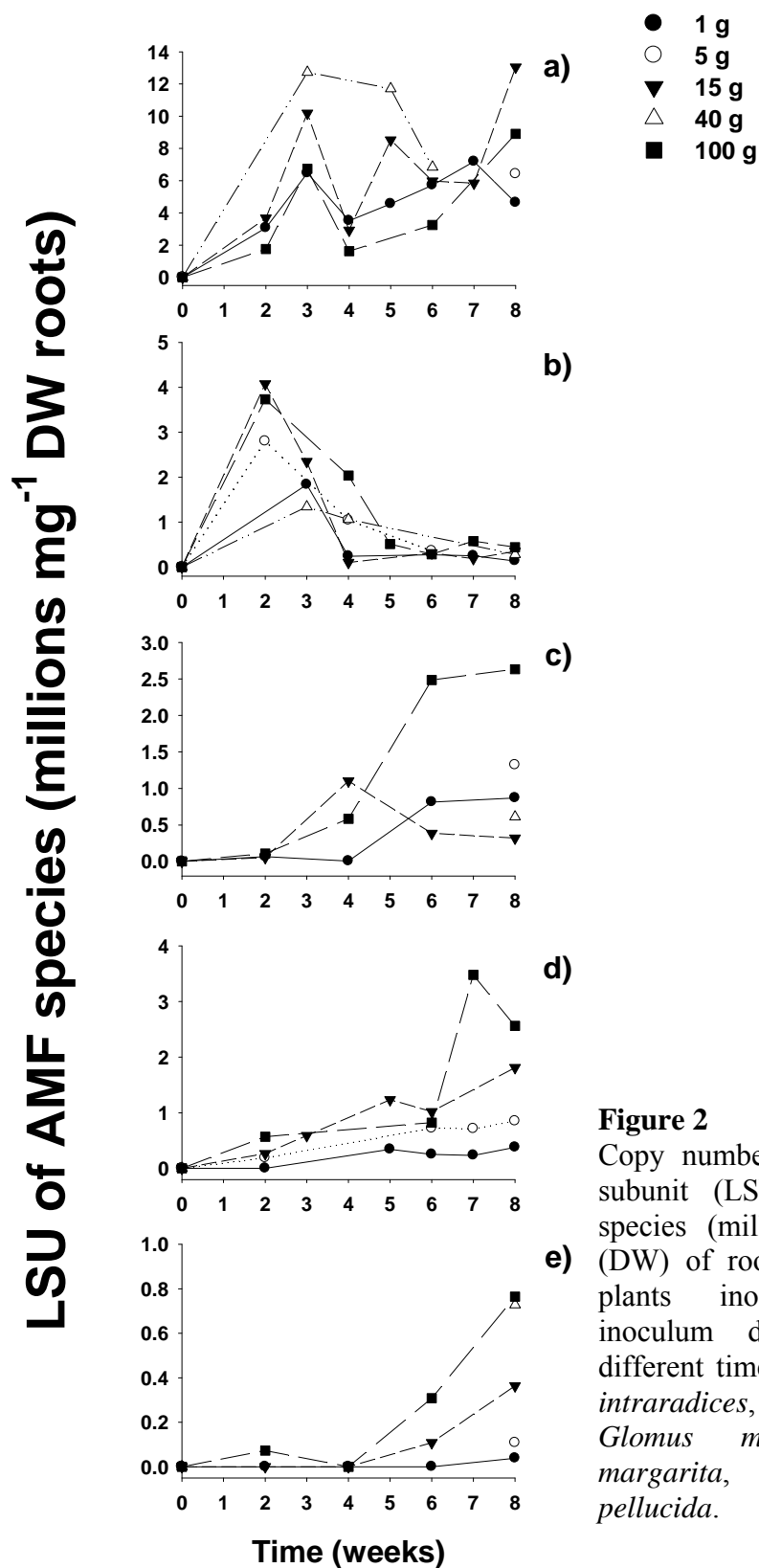


Figure 1

Double sigmoid regressions and associated R^2 values (**a**) of the fraction of medic root length colonized by AMF structures against the initial dose of AMF inoculum (g kg^{-1} substrate) and the time of harvest (weeks). The black dots are the raw experimental data. Section **b** shows sigmoid regressions of the temporal dynamics of the root colonization by the different AMF species split according to the different inoculation doses. Contour plots (**c**) show the relationships between the inoculum dose and the time of harvest for several arbitrary root colonization levels. Each root colonization level in these contour plots is accompanied by the 95%-confidence intervals (dashed lines), calculated using the standard errors of the parameters of the different models shown in section **a**.

Quantification of mycorrhizal LSU copy numbers by Real-Time PCR:

Another objective of this study was to develop quantitative tools, which would allow species-specific quantification of root colonization by the AMF, and to use them for assessment of root colonization by the AMF in the same samples, where the RLC levels were measured. The results are presented in Figure 2. For *Glomus claroideum* in particular, a peak of LSU concentration in the roots is observed at early harvests (2-3 weeks after sowing). The copy numbers then decline for *Glomus claroideum*, irrespective to the inoculum doses. For the other two *Glomus* species, some indications of maximum LSU concentrations early in the development were obtained, with some stagnation or decline in the later phases of development (Fig. 2). For *Gigaspora margarita* and *Scutellospora pellucida*, more or less steady increase in the LSU concentrations in roots was observed throughout the time. Especially for the two non-*Glomus* species, consistently higher LSU copy numbers were measured for treatments differing in the inoculum doses (Fig. 2). As a result, the relationship between the RLC and the LSU copy numbers per unit of root weight differed between the different AMF species. Those two measures of the mycorrhizal colonization have been plotted and correlated to each other (Fig. 3). Except for *Glomus claroideum*, the plots indicate a positive correlation between LSU copy numbers and the RLC with rather variable R^2 values. For *Glomus intraradices*, young roots with intermediate RLC values (~50%) are showing relatively high LSU copy number concentrations, similarly to older and more intensively colonized roots (as observed in the microscope). In total, the plot for this species shows a correlation pattern that differs for young and old roots.

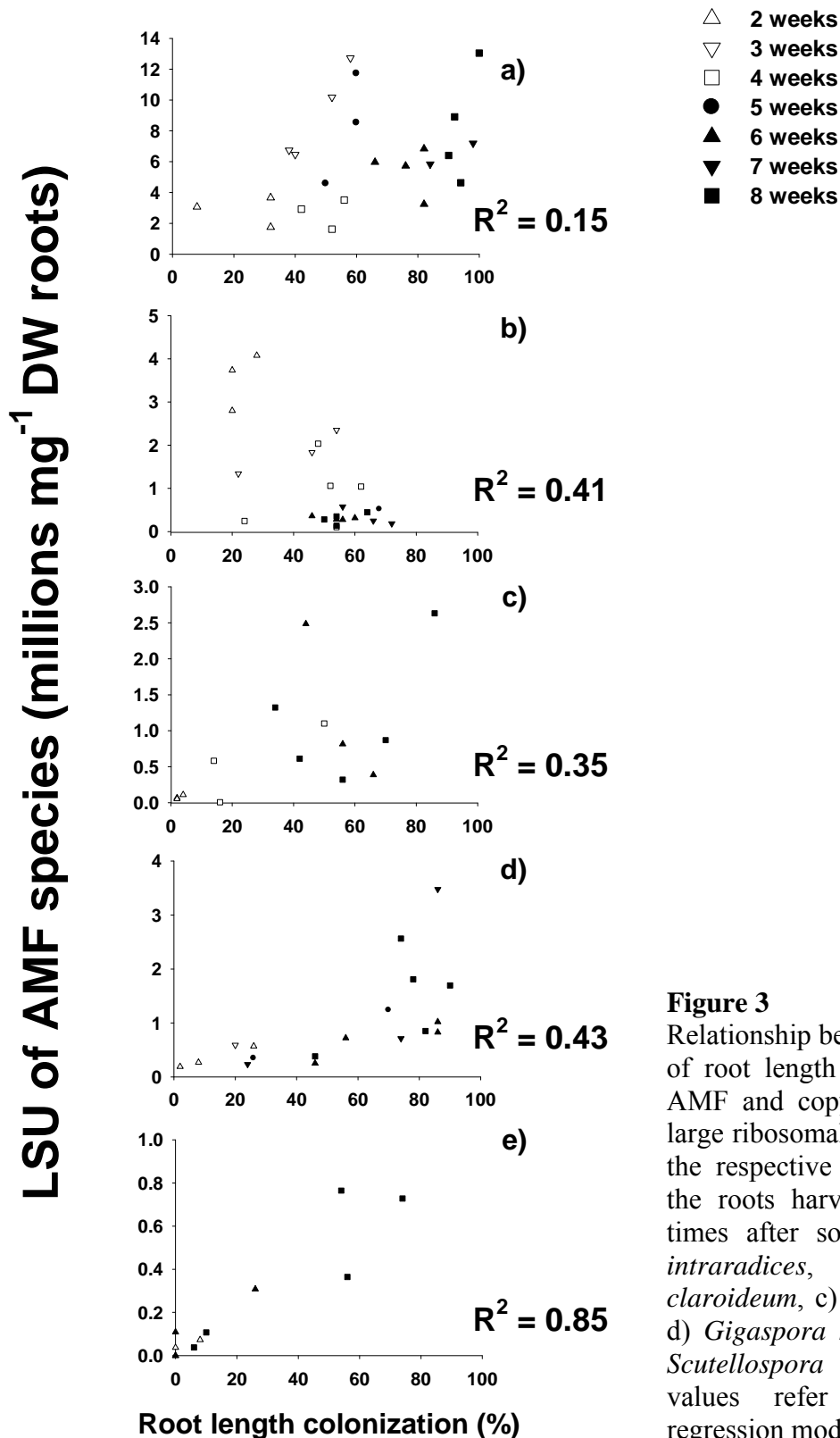
**Figure 2**

Copy numbers of the large ribosomal subunit (LSU) of 5 different AMF species (millions per mg dry weight (DW) of roots) in the roots of medic plants inoculated with different inoculum doses and harvested at different times after sowing. a) *Glomus intraradices*, b) *Glomus claroideum*, c) *Glomus mosseae*, d) *Gigaspora margarita*, and e) *Scutellospora pellucida*.

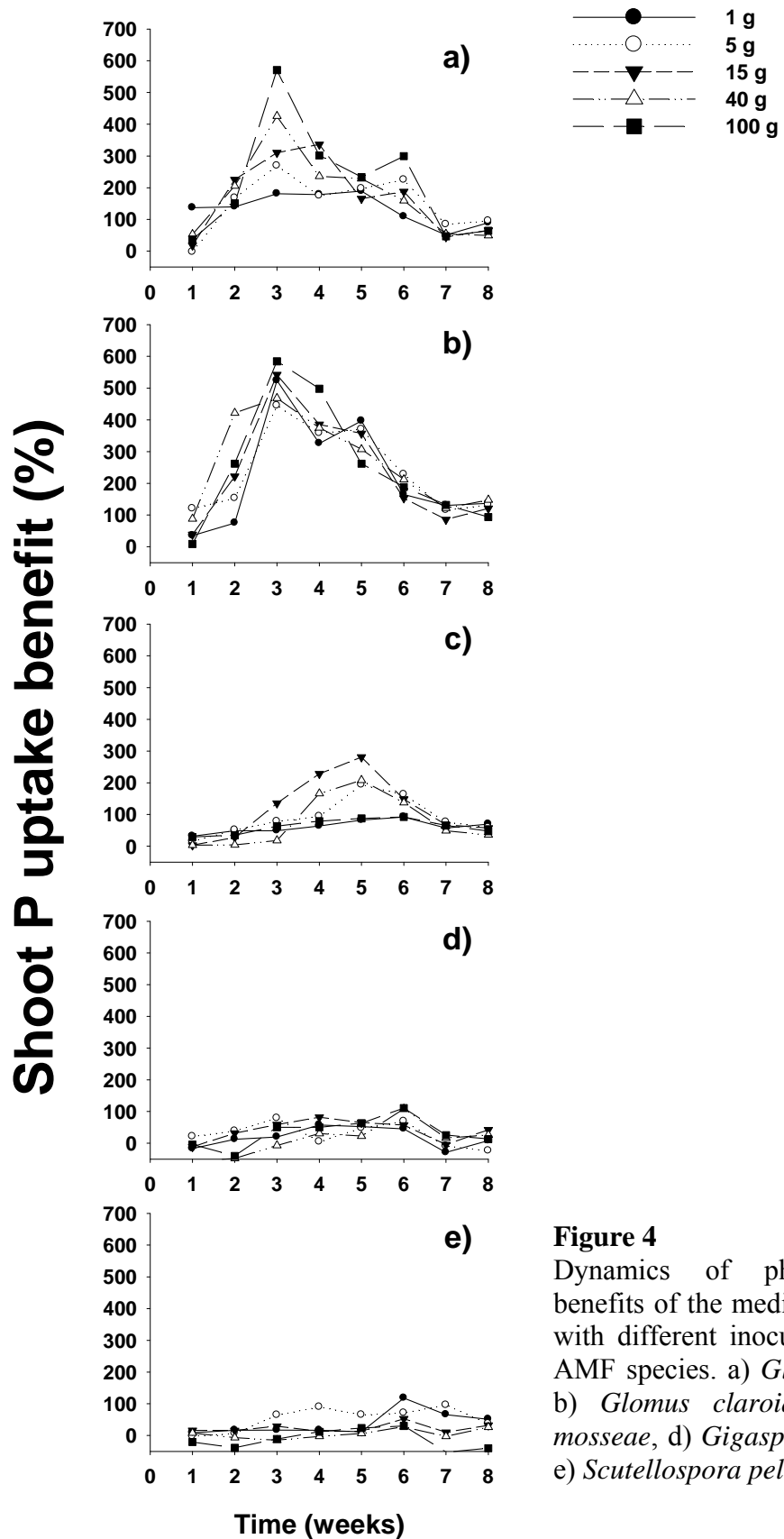
The situation for *Glomus claroideum* is particular in the sense that it shows a negative correlation between the LSU copy numbers and the RLC values measured by microscopy. Young roots (2 weeks) exhibit high numbers of LSU copies per unit of root weight, while they are still poorly colonized (as assessed by microscopy) and older roots that are well colonized as for the microscopy, see their content of LSU copy numbers heavily reduced. The situation is less clear for *Glomus mosseae* but the LSU copy numbers generally increase with increasing RLC values. For *Gigaspora margarita* and *Scutellospora pellucida*, the positive correlation between the two colonization measurements is rather significant, and for *Gigaspora margarita* the relationship appear to follow a power rather than a linear function (Fig. 3).

Plant responses to mycorrhizal colonization:

Plant biomass and shoot phosphorus concentrations have been measured for each plant. For individual mycorrhizal treatments, the results are collectively presented as the shoot P uptake benefits (Figure 4). These results indicate strong P benefits at early developmental stages (3-4 weeks after inoculation) for *Glomus intraradices* and *Glomus claroideum* with values reaching up to 600%. These early shoot P uptake benefits appeared to correlate with the applied inoculum dose for *Glomus intraradices* at 3 weeks after sowing, whereas there were no clear trends with respect to the inoculum dose for *Glomus claroideum*. Afterwards, the shoot P uptake benefits decreased for both species, but the mycorrhizal plants were still containing about twice as much P as the non-mycorrhizal plants of the same age (Fig. 4). For *Glomus mosseae*, the calculated shoot P uptake benefits were also reaching a maximum in the middle of the experiment, but comparably later (5 weeks) than for the two other *Glomus* species. Interestingly, the two most extreme inoculum doses (1 and 100 g kg⁻¹) did not exhibit much P uptake benefit to the plants at any time. For *Gigaspora* and *Scutellospora*, the P uptake benefits were generally much lower than for any of the *Glomus* species, peaking later in the ontogeny (6 or more weeks after sowing). Some plants showed even negative P uptake benefits, meaning that they contained less P than the shoots of the non-mycorrhizal plants (Fig. 4).

**Figure 3**

Relationship between the fraction of root length colonized by the AMF and copy numbers of the large ribosomal subunit (LSU) of the respective fungal species in the roots harvested at different times after sowing. a) *Glomus intraradices*, b) *Glomus claroideum*, c) *Glomus mosseae*, d) *Gigaspora margarita*, and e) *Scutellospora pellucida*. R^2 values refer to the linear regression model.

**Figure 4**

Dynamics of phosphorus uptake benefits of the medic plants inoculated with different inoculum doses of five AMF species. a) *Glomus intraradices*, b) *Glomus claroideum*, c) *Glomus mosseae*, d) *Gigaspora margarita*, and e) *Scutellospora pellucida*.

Discussion:***Root colonization assessed by microscopy: dose and time responses***

The fraction of root length colonized (RLC), assessed by traditional staining and microscopy approach, confirmed an expected S-curve time response for the five AMF tested. This is fully in accordance with the results found in the literature for those AMF species (Hart & Reader, 2002c; Jansa *et al.*, 2008), where sequential harvests were carried out to follow the progress of root colonization. The maximum level (or plateau) of colonization achieved over time varied among the different AMF species and this confirmed the differential infectivity potentials of the different AMF species, as observed in other studies (Hart & Reader, 2002c; Munkvold *et al.*, 2004; Hart & Reader, 2005; Avio *et al.*, 2006). Specifically, the maximal RLC of *Glomus claroideum* colonizing medic roots was about 60%, in contrast to *G. intraradices*, which filled nearly all roots at final harvest – and this was in a good agreement with previous observations (Jansa *et al.*, 2008). For Wilson and Trinick (1983), the infection development by a single fungus is influenced by inoculum density, whose augmentation will decrease the lag phase before infections commences and thereby increases the rate at which infection develops. This situation is only observed for the non-*Glomus* species (*Gigaspora margarita* and *Scutellospora pellucida*) in this experiment. The fact that the dose had almost no influence on the RLC for the three *Glomus* species could be explained by (1) the presence in their inocula of additional infective propagules (colonized root fragments, old mycelium), and/or (2) by the capacity of these species to propagate their root colonization quickly within the root. The non-spore propagules are known not to be source of infection for the other two species (Klironomos & Hart, 2002). This is also supported by the findings of Hart and Reader (2002c) who observed that AMF regenerating primarily from spores (i.e. members of the Gigasporaceae) are the slowest colonizers independently of the used inoculum density.

Quantification of AMF gene copy numbers by Real-Time PCR:

A first observation concerns the range of the LSU copy numbers that differs for the different AMF species. While the LSU copy numbers measured for *Glomus claroideum*, *Glomus mosseae* and *Gigaspora margarita* are in the same range, we observed that those measured for *Glomus intraradices* and *Scutellospora pellucida* are on average in a lower

range than the others (Fig. 2). This could partly be explained by possible differences in colonization intensity, which is a feature commonly missing in the traditional colonization estimates (e.g. numbers of hyphae crossing one root intersection). On the other hand, these results may indicate that different concentrations of nuclei are present in the same biomass of the different AMF species. However, experimental evidence for this is missing. What is known is that the number of nuclei per AMF spore is varying for different AMF species as well as the DNA content per nucleus (Hosny *et al.*, 1998). Nevertheless that kind of estimations calculated per mycelium biomass or length is missing and that studies that have been carried out on nuclei in hyphae are rather of qualitative than of quantitative nature (Bago *et al.*, 1998; Bago *et al.*, 2002).

Concerning the dynamics of the LSU copy numbers measured in roots at different harvests, the most outstanding result is the varying pattern for the different AMF species and more specifically the peak measured after 2 weeks in roots colonized by *Glomus claroideum* (Fig. 2) and the maximum observed at the same time for *Glomus intraradices*. In previous study (Jansa *et al.*, 2008), colonization of the same *Glomus* isolates was measured in medic and leek roots after 4 and 8 weeks with a similar approach, employing both staining-microscopy and quantitative PCR. Those previous results revealed important numbers of LSU copy for *Glomus mosseae* compared to *Glomus claroideum* and *Glomus intraradices* after 4 weeks (almost 10 times higher in medic roots) followed by an abrupt decline after 8 weeks to reach copy numbers in the same range of those measured in roots colonized by *Glomus claroideum* and *Glomus intraradices*. A similar maximum of ribosomal DNA has been observed in the study of Isayenkov *et al.* (2004) for *Glomus intraradices* while the RLC measured by microscopy was still progressing. The soil properties (plant available P, pH, substrate texture etc.), the climatic conditions, the number of harvests, the molecular assay and the inoculum density in these experimental set-up were different but nevertheless these studies including the present one revealed that LSU copy numbers for fast colonizers may reach a maximum much earlier than the root colonization assessed by microscopy.

The observed decline in LSU copy numbers measured in roots colonized by *Glomus claroideum* and *Glomus intraradices* after 2 and 3 weeks respectively might be explained by a rapid spread of active mycelium, followed by a decrease in vitality of the

colonization – either some of the hyphae die or their metabolic activity ceases, which may result in reduction of nuclear counts per unit of mycelium length/mass. Jansa *et al.* (2008) proposed that multiplication of nuclei within the hyphae of (some) AMF may occur at time of peak metabolic activities. Afterwards, the diminution in vitality of colonization is likely to impact the number of nuclei per fungal biomass whose decrease could result from a dilution of the nuclei in the developing hyphae or from occurrence of senescing hyphae inside the roots. Vital staining of intraradical hyphae has previously allowed showing a decline in AMF activity in colonized roots after some times (Smith & Dickson, 1991; Tisserant *et al.*, 1993; Vierheilig *et al.*, 2005). Together, these phenomena would result in a discrepancy between DNA quantification and staining as measures of fungal biomass, the latter estimating both living and dead fungal structures. Furthermore, the traditional microscopy based approach usually misses the intensity of colonization, and this compromises the use of this approach as a proxy for AMF biomass estimation in roots. Interestingly, the maxima observed for the LSU copy numbers occurred when *Glomus claroideum* was almost reaching its maximum of RLC of 60% while the RLC measured for *Glomus intraradices* was still in progress (Fig. 1b) to reach its maximum of 90% at late harvests, where a second (less prominent) increase of LSU copy numbers was observed (Fig. 2a). We can speculate that the roots colonized by *Glomus claroideum* at a certain degree get “self-immunized” to further colonization by the same AMF isolate and possibly by other phylogenetically related AMF species (see chapter 3; competition between *Glomus claroideum* and *Glomus intraradices*). At the same time, roots colonized by *Glomus intraradices* seem to be able to accommodate more of this fungus which is translated by the progress of its RLC and by the second peak of measured LSU copy numbers.

Phosphorus uptake benefits to mycorrhizal colonization

It used to be thought that the AMF colonizing extensively the root system of their host would provide important benefits in terms of plant growth and P acquisition (Abbott & Robson, 1981). It is now well accepted that it is rather the ability of AMF species to extensively colonize the substrate than to colonize the roots that contributes to a better plant response (Smith *et al.*, 2004; Avio *et al.*, 2006; van der Heijden & Scheublin, 2007) regarding these attributes. In this experiment, plants inoculated by *Gigaspora margarita*

and *Scutellospora pellucida* can accommodate respectively up to 80 and 60% of RLC (Fig. 1) but the P uptake benefits are relatively low compared to those resulting from root colonization by the *Glomus* species (Fig. 4). These issues will be discussed in the next chapters.

Nevertheless, the results presented in Fig. 4 and especially the peaks observed after 3 weeks for both *Glomus intraradices* and *Glomus claroideum* would support the previous idea that the peaks of LSU copy numbers measured for these two species are associated with a peak of their metabolic activities, acquisition of phosphorus being the major function of the mycorrhizal symbiosis. In the study of Jansa *et al.* (2008), high numbers of LSU copies measured in medic roots colonized by *Glomus mosseae* after 4 weeks were also associated with a better P uptake compared to roots colonized by *Glomus intraradices* and *Glomus claroideum*.

All these information are summarized in Figure 5 where the different regression models elaborated on the raw data (for RLC, RLC by arbuscules, P uptake benefit) allowed comparing the different AMF species for (hypothetical) equivalent density of spores in the pots. It shows the diversity of root colonization ontogenies and of the resulting plant responses concerning the P acquisition. In Figure 5b, it is interesting to note that for *Glomus claroideum*, the maximum of root length colonized by arbuscules is achieved relatively early (after 3 weeks, then followed by a small decrease over time not observable on the model but well with the raw data), supporting again the idea that the root colonization assessed by quantitative PCR reflects well the metabolically active fungal biomass inside the roots. Diversity of root colonization ontogenies was also coupled to a diversity of plant ontogenies. Indeed, in this experiment, plants colonized by the different AMF species were presenting different growth forms (see Figure 6) and more interestingly, specific events of plant development like flowering, pods formation and senescence were occurring differently according to the mycorrhizal treatment. Representation of ontogenetic events for a plant colonized by *Glomus claroideum* is presented in Figure 7a. Compared to non-mycorrhizal treatments, flowering, pods formation and senescence occurred much earlier (data not shown). On the other side, plants colonized by *Gigaspora margarita* and *Scutellospora pellucida* were experiencing these events later than the non-mycorrhizal plants (data not shown). These events are

important factors for the plant fitness (producing offspring that will survive and further reproduce), and the ecological meaning of the differential influence of mycorrhization on these features is largely unknown. As a consequence, when comparing plants colonized by different AMF, we should consider comparing plants of the same growth (or developmental) stage instead of the same age. This is not an easy task, especially if we also take into account the fungal development (spore germination, root infection, load of carbon from the root etc) whose ontogeny is also varying between the different species. This is probably a criticism that can be addressed to the recent study by Gamper *et al.* (2008) that have compared fungal biomass and its ribosomal DNA and RNA contents for fungal structures of completely different ages.

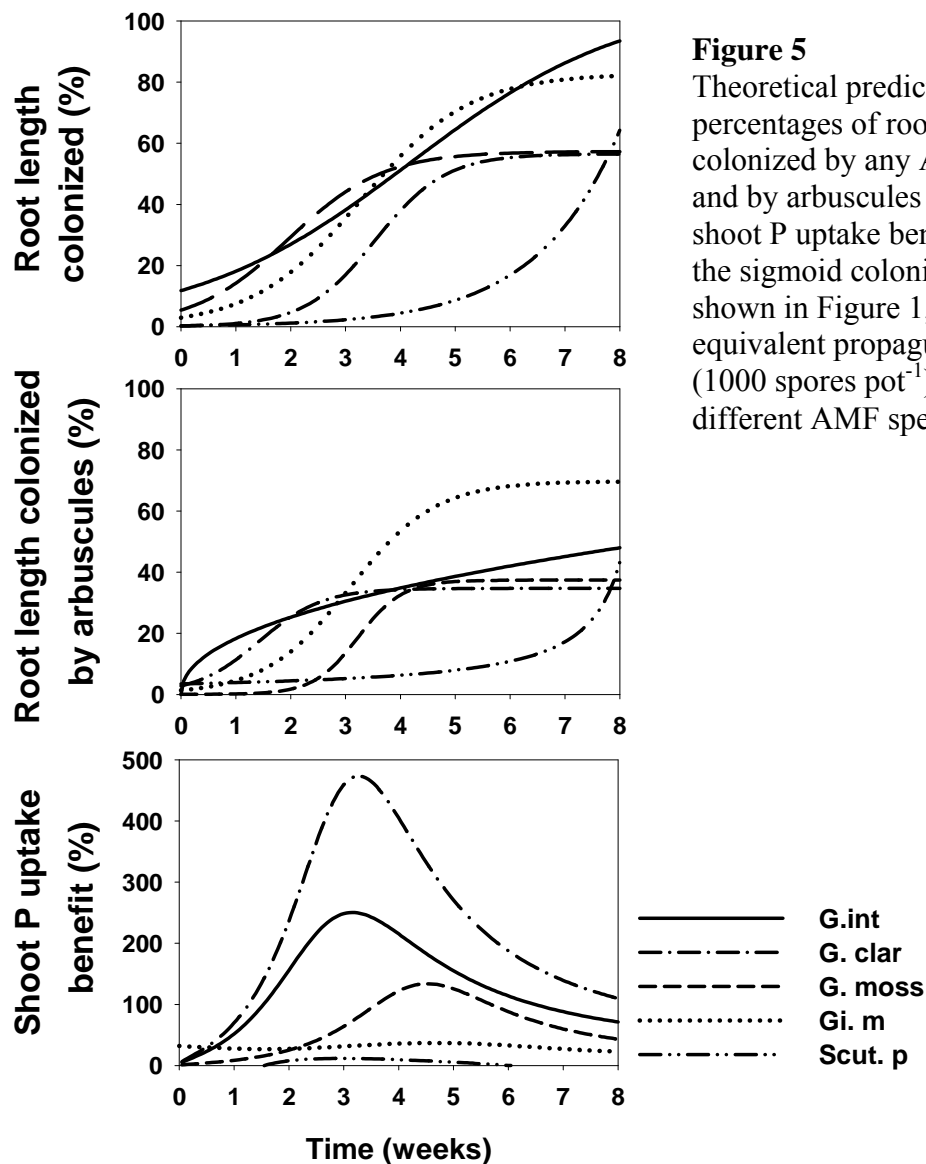


Figure 5

Theoretical predictions of percentages of root length colonized by any AMF structures and by arbuscules only, and the shoot P uptake benefits based on the sigmoid colonization models shown in Figure 1, assuming equivalent propagule densities ($1000 \text{ spores pot}^{-1}$) of the five different AMF species

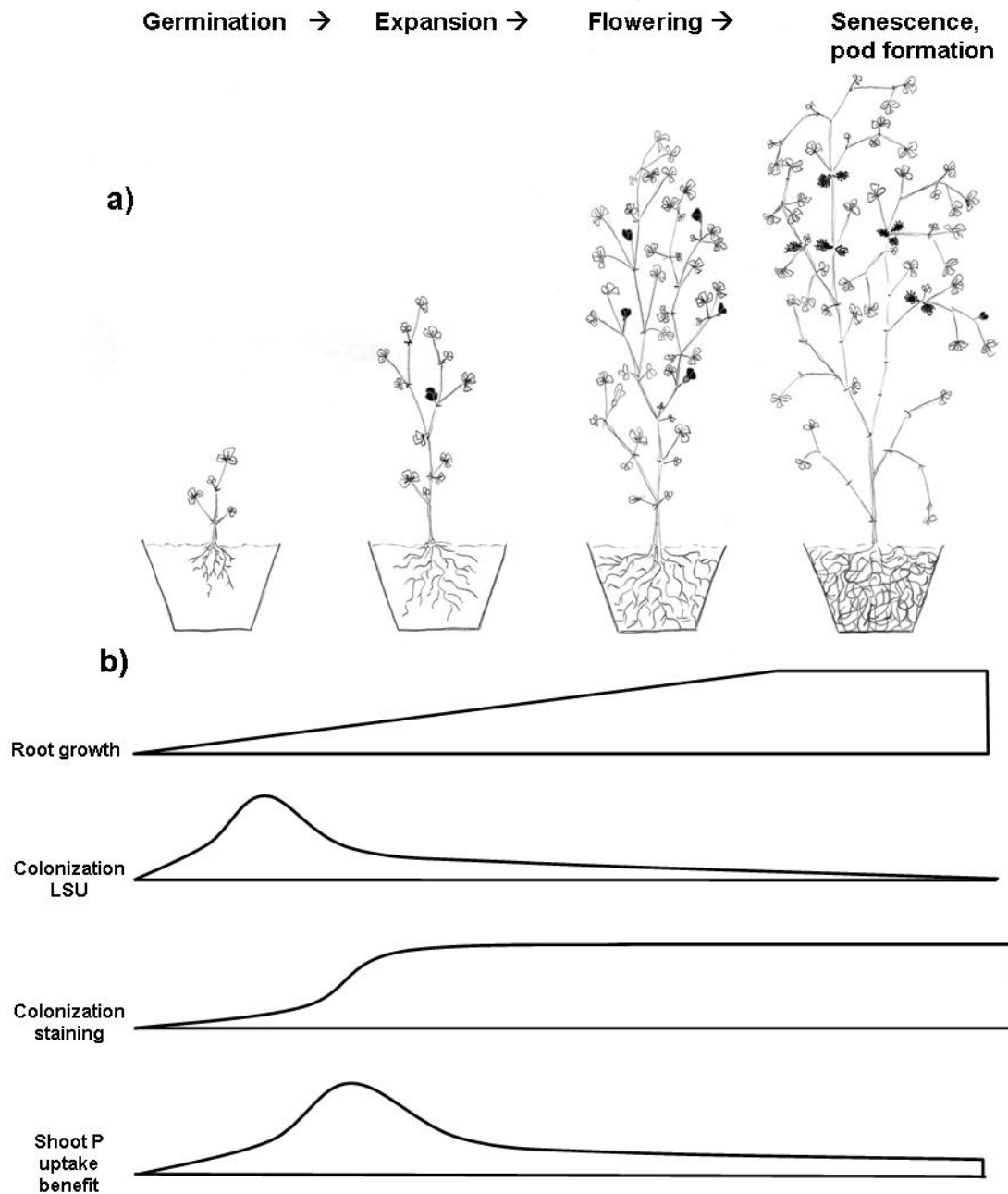
Conclusions:

- RLC assessed by microscopy does not always correlate with the gene copies of the AMF species as assessed by real-time PCR.
- Quantitative PCR proved to be a very useful tool for quantification of root colonization (objective, easy, fast).
- Quantification of ribosomal RNA instead of ribosomal DNA could represent another alternative as suggested by Jansa *et al.* (2008) and Isayenkov *et al.* (2004). This quantification is probably more able to reflect the activity of the fungus when colonizing roots. The potential of quantifying mRNA of functional genes needs to be revealed yet. Care should be taken when comparing plants of different ages colonized by different AMF species that may differentially modify plant ontogeny with unknown consequences on the fungal development.



Figure 6

Medic plants inoculated with non-mycorrhizal inocula (NM) or with one of five different AMF species (100 g inoculum kg⁻¹ substrate), 4 weeks after sowing

**Figure 7**

Schematic representation of the main ontogenetic events of the pot-grown medic plant (**a**), and the root colonization by *Glomus claroideum* assessed by different approaches and the mycorrhizal P uptake benefits to the plants (**b**).

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Chapter 2: Diversity of phosphorus acquisition and use efficiency in *Medicago truncatula* colonized by different mycorrhizal fungi

Abstract:

Diversity in phosphorus (P) acquisition strategies was assessed among three species of arbuscular mycorrhizal fungi (AMF) isolated from a single field in Switzerland. *Medicago truncatula* was used as a test plant and a compartmented system coupled with dual radioisotopes labeling (^{32}P and ^{33}P) employed to characterize the contribution of the morphological and physiological traits of the external mycorrhizal mycelium to the P acquisition by the plants. *Glomus intraradices*, *Glomus claroideum* and *Gigaspora margarita* were able to take up and deliver P to the plants from maximal distances of 10, 6 and 1 cm from the roots, respectively. *Glomus intraradices* most rapidly colonized the available soil volume and transported significant amounts of P towards the roots, but provided the same growth benefit as compared to *Glomus claroideum*, whose mycelium was shown to be less efficient in soil exploration and in P uptake and delivery to the roots. These differences are probably related to different carbon requirements by these different *Glomus* species. *Gigaspora margarita* provided only limited P benefits to the plants. It accessed only a limited soil volume, where it established dense mycelium networks, within which the P was most probably immobilized before being delivered to the roots. Numerical modeling identified possible differences in growth patterns of extraradical mycelium of the different AMF species. Namely, hyphal turnover was pointed out as important factor governing hyphal network development in *Gigaspora*, whereas hyphal anastomoses and apical branching were key features for *G. intraradices* and *G. claroideum*, respectively.

Keywords:

Arbuscular mycorrhiza; Costs and benefits; Extraradical mycelium, Functional diversity; *Medicago truncatula*; Phosphorus

Introduction:

The majority of land plant species form symbiotic associations with the arbuscular mycorrhizal fungi (AMF). These mutualistic associations play an important role in plant nutrient uptake and in environmental stresses tolerance and often result in a better plant growth and nutrition, particularly under nutrient deficient conditions (Smith & Read, 1997). In addition, AMF communities are involved in many ecosystem processes, influencing the soil structure and the plant community composition (Read & Perez-Moreno, 2003). The outcome of the symbiosis can range from negative to positive plant growth benefits and depends mainly on the identity of the plant and AMF species and on the environmental conditions (Burleigh *et al.*, 2002; Smith *et al.*, 2004).

During root colonization, external mycelium is produced in the surrounding soil, which can take up the inorganic phosphate from the soil solution. This is further translocated to the host root and delivered to the plants at the root-arbuscule interface. These complex processes imply the action of different transporters and enzymes (of plant and fungal origin), some of which are already known at gene level (see Javot *et al.* (2007) for a review). Upon establishment of AMF colonization, plants can acquire phosphorus (P) both at the soil-root interface through root epidermis and root hairs (root uptake pathway) and through mycorrhizal mycelium in soil (mycorrhizal uptake pathway). In some cases, it has been shown that plants could derive all their P from the mycorrhizal uptake pathway even if no net benefit in terms of P content or plant biomass were observed (Smith *et al.*, 2003; Smith *et al.*, 2004).

In exchange of the P uptake mediated by the fungus, AMF receive from the plant carbon compounds derived from the photosynthesis. However, as compared to the P uptake benefits, only very few studies have analyzed the real costs of the mycorrhizal symbiosis. It has been estimated that the symbiosis with AMF can cost the plant up to 20% of the plants net photosynthates (Jakobsen & Rosendahl, 1990) but some reports indicate important variation in the C costs depending on individual plant-fungus species combinations, amount of fungal biomass produced and also on environmental conditions (Jakobsen *et al.*, 2002; Lerat *et al.*, 2003; Munkvold *et al.*, 2004).

Important functional differences in term of P acquisition strategies have been recognized among AMF species and also among AMF isolates belonging to the same species. These

are mainly expressed as: 1) morphological traits such as the ability (rate and extent) of the AMF to colonize the root and the soil and 2) physiological traits that mainly include the efficiency of the mycorrhizal pathway to take up the P, transport and deliver it to the roots along with the carbon requirement from the plant host (van der Heijden & Scheublin, 2007). There is a consensus (Avio *et al.*, 2006; van der Heijden & Scheublin, 2007) that the differential increases in P supply to host plants are mainly attributed to phenotypic and functional properties of the extraradical mycorrhizal mycelium (ERM). The phenotypic properties refer here to the ability of the ERM to be extensive, viable and interconnected through anastomoses, whereas the functional properties depend, among others, on the levels of expression of P transporters and other proteins involved in the P transport and delivery to the roots. Together, these morphological and physiological traits may be modulated by the host plant species and the environmental conditions (van der Heijden & Scheublin, 2007).

To further characterize the levels of functional diversity of AMF at a single field site, previous studies focused solely on *Glomus* species (Jansa *et al.*, 2005; Jansa *et al.*, 2008) isolated from the Tännikon field site (Jansa, J. *et al.*, 2002) are now amended by another isolate belonging to the family of *Gigasporaceae*. In this study we aimed at quantification of differences in P acquisition and use efficiency of medic (*Medicago truncatula* Gaertn.) when colonized by three different AMF species (*Glomus intraradices*, *Glomus claroideum* and *Gigaspora margarita*) that were isolated from the same field in Switzerland (Jansa *et al.*, 2002). The plant responses (P uptake and plant growth) were analyzed with regards to (i) the ability of the fungal symbionts to colonize the substrate, considering the rate and extent of the colonization, (ii) the efficiency of P uptake and transport towards the plants by the fungi, and (iii) the P use efficiency (g mg^{-1}) of the mycorrhizal plants. Results provided by the analysis of the extent of substrate colonization were fitted to the growth model of AMF developed by Schnepf *et al.* (2008) in order to identify the differences in mycelium growth patterns by the different AMF species. We have employed a compartmented system consisting of a plant container and a root-free zone, and we used ^{32}P and ^{33}P radioisotope tracing so as to estimate the magnitude and rates of P fluxes between the fungi and the plants. P use efficiency was used as a proxy for the C costs of the symbiosis.

Materials and methods

Experimental setup

The experiment was carried out in cuvettes having plant (15×15×4 cm) and root-free (15×15×11.1 cm) compartments. ^{33}P and ^{32}P isotopes were applied in the latter (Fig 1). All zones of the cuvettes were filled with a substrate consisting of sterilized field soil, coarse quartz sand (partical diameter 0.7-1.2 mm) and fine quartz sand (grain diameter 0.08-0.2 mm), mixed in the ratio of 1:3:1 (v:v:v). The soil was collected in Tänikon, Switzerland, air-dried, passed through 5 mm sieve and γ -irradiated at Studer Hard, Däniken, Switzerland, applying a dose of 25 – 75 kGy with a ^{60}Co source. The available P content of the substrate was $21.9 \pm 0.43 \text{ mg kg}^{-1}$ (ammonium acetate-EDTA extraction, 1:10 w:v, 16h), and the readily available P pool ($E_{1\text{min}}$) was $1.73 \pm 0.06 \text{ mg kg}^{-1}$ as assessed by the isotope exchange kinetics approach (Frossard & Sinaj, 1997). Substrate C and N contents were 2.2 ± 0.1 and $0.24 \pm 0.01 \text{ g kg}^{-1}$, respectively. Plant roots were confined into the plant compartment by a 30 μm mesh (Fig. 1b). Root-free zone consisted of four compartments, separated from each other by 500 μm meshes. The two buffer zones and ^{32}P labeling zone were the same for all cuvettes, whereas the distance between the plant and the ^{33}P labeling zone varied at five levels between 1.1 and 9.1 cm (Fig. 1a). Four cuvettes were prepared for each of the five distances between the plant and ^{33}P labeling compartments. Before planting, the ^{33}P labeled compartment was filled with the substrate labeled with radioisotope solution (1 ml of aqueous $^{33}\text{PO}_4^{3-}$ solution; carrier-free orthophosphate, Hartmann Analytic GmbH, Braunschweig Germany; 4.14 MBq ml^{-1}). Forty eight hours before harvest, 3 ml of aqueous solution of $^{32}\text{PO}_4^{3-}$ (0.5 MBq ml^{-1}) were injected into the ^{32}P labeling zone at a distance of 0.9 cm from the plant compartment (Fig 1c). Three quick injections of 1 ml each were used to deliver the desired amount of ^{32}P isotope into the labeling zone. The needle used for the ^{32}P labeling was 15 cm long and was designed so as to maximize homogeneity of the labeling (Fig 1d).

Experimental design

One host plant and four different inoculation treatments were considered, including one non-mycorrhizal control and three AMF treatments. Five replicate cuvettes were established for each inoculation treatment, each of them with a different distance between the plant and ^{33}P labeling zones. This resulted in completely randomized design with 5

replicates with respect to all variables except ^{33}P acquisition by plants, for which this experiment presented a regression design with 5 treatment levels (distances).

Plants and AMF

Seeds of *Medicago truncatula* Gaertn. (medic) were surface-sterilized for 10 min in concentrated (97%) sulphuric acid (Massoumou *et al.*, 2007) and then washed in sterile water and germinated for 3 days on moistened filter paper. Three seedlings were planted per plant compartment of each cuvette at the start of the experiment. Plants were grown in the cuvettes for 42 days.

Three AMF species, all isolated from a single field site in Switzerland (Jansa, J. *et al.*, 2002), were used in this study. These were *Glomus intraradices* BEG 158, *Glomus claroideum* BEG 155 and *Gigaspora margarita* BEG 152. The inoculum was produced in 1 kg pots filled with the substrate described above. Inoculum pots were planted with leek plants and grown for 10 months. The density of the AMF spores was assessed in the inoculum following wet sieving and sucrose density-gradient centrifugation. Based on previous infectivity assays (chapter 1), the inocula of the three AMF species were diluted so as to achieve 50 % root length colonized after 6 weeks of growth, for each inoculation treatment. Thus the inocula were diluted with sterile potting substrate so as to reach 500, 1000 and 15000 spores per kg of substrate dry weight for *Glomus intraradices*, *Glomus claroideum* and *Gigaspora margarita*, respectively. Non-mycorrhizal cuvettes were inoculated with substrate where non-mycorrhizal leek was grown for previous 10 months. Plant compartments were watered every day and root-free zone every other day. The plants received 50 ml cuvette⁻¹ week⁻¹ of a modified Hoagland nutrient solution (Hoagland & Arnon, 1950) containing no P throughout the duration of the experiment. The cuvettes were completely randomized in a growth chamber (Conviron PGV36, Winnipeg, Canada) under the following conditions: temperature 22/19°C and relative aerial humidity 75/90% (day/night, respectively), photoperiod 16 h, combined fluorescent and incandescent light, 330 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Rate and extent of substrate colonization by the mycorrhizal mycelium were assessed by Geiger-Müller counter (Figure 2 and table 2 for details).

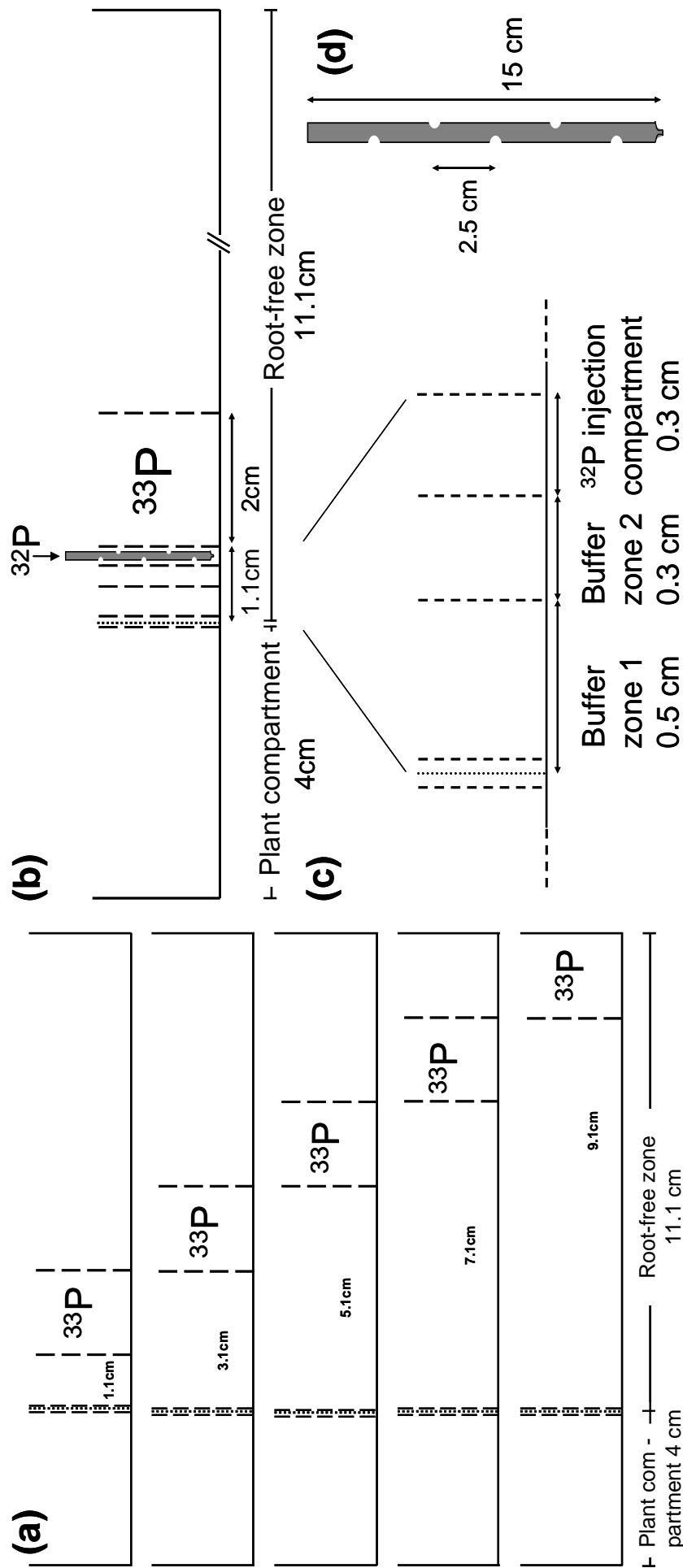
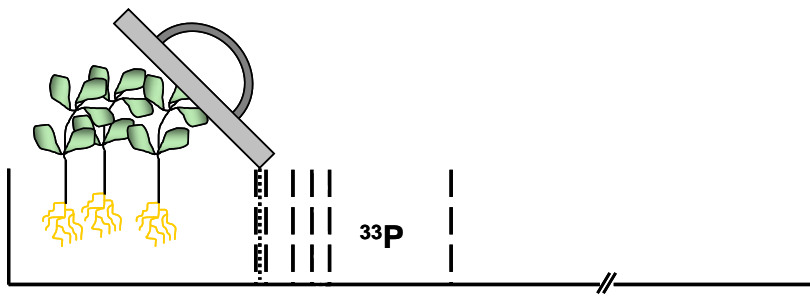


Figure 1

Scheme of the cuvette used for plant cultivation and substrate labeling. Dashed lines – 500 μm mesh, dotted lines – 30 μm mesh. The ^{33}P labeled substrate was added to the system in a 2 cm slice placed at an increasing distance from the plant compartment (a). The plant compartment was separated from the root-free zone by a sandwich of three meshes (b). The ^{32}P labeled solution was injected 48 h before the harvest in a compartment separated from the plants by two buffer zones (c). Representation of the 15 cm long needle used for a homogenous labeling of the substrate with ^{32}P solution (d).

**Figure 2**

Non-destructive recording of radioactivity in plant shoots with the hand-held Geiger-Müller monitor.

Harvest and measurements

Shoots, roots and substrate from the plant compartment were harvested for further analyses. In the root-free zone, all substrate was collected from the buffer zone 1 (Fig 1c) and used to isolate the mycelium that had grown in this zone. This was done by wet sieving through 500 μm and 40 μm sieves and by multiple decanting of the fraction collected on the finer sieve. Representative substrate samples were taken from following distances from the root barrier at each root-free zone: 0.25 cm (buffer zone 1), 0.95 cm (^{32}P injection compartment), 2.1 cm, 4.1 cm, 6.1 cm; 8.1 cm and 10.1 cm. Plant shoots were dried at 105 °C for 48 h and weighed. Roots were washed from the substrate under tap water and then rinsed with deionized water and cut to about 1 cm pieces and mixed. Subsamples were taken for dry matter assessment and root staining. The roots staining procedure was following the protocol described by Phillips and Hayman (1970) and Brundrett *et al.* (1984). Briefly, roots were macerated in 10% KOH (100g l⁻¹) at 90°C for 25 min, rinsed with water, incubated in 1% HCl (10g l⁻¹) at room temperature for 1 h, briefly rinsed with water before transfer to 0.05% Trypan blue (0.5 g l⁻¹) in lactic acid: glycerol: water (1:1:1; v:v:v). In this solution, the roots were stained at 90°C for 2 h and then at room temperature overnight. Finally, the roots were de-stained for at least 24 h in water at room temperature. The extent of root length colonized by hyphae, arbuscules and vesicles was determined on stained roots according to the method of McGonigle *et al.* (1990), recording 50 intersections per sample. Dried root and shoot biomass as well as mycelium extracted from buffer zone 1 (200 – 500 mg per sample) were incinerated at 550°C for 8 h and ashes dissolved in 2 ml HNO₃ 65% (650g l⁻¹), made to 25 ml with

distilled water and filtered through a paper filter (Whatman No 40). The concentrations of P and activity of both ^{33}P and ^{32}P isotopes were determined in the extracts according to Ohno and Zibilske (1991) and by scintillation counting, respectively. The radioactivity of the two radioisotopes in each sample was assessed on a Packard TR 2500 liquid scintillation counter, using energy separation (lower energy window 2-300 keV, upper energy window 301-1700 keV) and 10 min counting per sample. Scintillation counting protocol was calibrated for dual ^{33}P and ^{32}P measurements with automatic quenching correction using single radioisotope solutions and chemical quencher (CCl_4). Approximately 3 grams of substrate collected in plant compartment and at different distances from the plants were used for estimation of hyphal length density (HLD) as described before (Jansa *et al.*, 2003a).

Calculations and statistics

Phosphorus concentrations in plant extracts were used for calculation of plant P content. Plant P uptake from the substrate was determined by the subtraction of P contained in the medic seeds (average of $19.5 \mu\text{g seed}^{-1}$) from total plant P content at harvest. Plant growth and P uptake benefits were calculated according to Cavagnaro *et al.* (2003), using individual biomass and P uptake values of inoculated plants (Mb and Mp, respectively) and means of the biomass and P uptake values of non-mycorrhizal plant (NMb and NMp, respectively; means of five replicates):

$$\text{Plant growth benefit (\%)} = \frac{Mb - NMb}{NMb} \times 100 \quad (\text{Equation 1})$$

$$\text{P uptake benefit (\%)} = \frac{Mp - NMp}{NMp} \times 100 \quad (\text{Equation 2})$$

Phosphorus use efficiency (g mg^{-1}) was calculated by dividing the plant dry mass by the plant P content (Baon *et al.*, 1993). Percentages of ^{32}P and ^{33}P transported to the plant were calculated as ratio of radioactivity of the specific isotope in the plant (roots and shoot combined), divided by the amount of the isotope administered per cuvette. Decay correction was employed to compare scintillation counting readings from the plant and fungal biomass to the activity of the labeling solution (measured before application). The mycorrhizal plant P uptake pathway efficiency was calculated by dividing the ^{32}P activity (kBq) recovered in the plants (shoot and roots combined) by the HLD (m g^{-1}) measured

in the ^{32}P injection compartment. Specific ^{33}P and ^{32}P activities were calculated by dividing the radioactivity of a specific isotope (corrected for decay) by the P content of the plant. The statistics (ANOVA, multiple range tests) were calculated in Statgraphics *Plus* for Windows version 3.1.

Hyphal growth model

Measured hyphal length densities in the substrate at different distances from plants were analyzed using the growth model for arbuscular mycorrhizal fungi of Schnepf *et al.* (2008) in order to identify possible differences in mycelium growth patterns by the different AMF species. The model simulates the dynamic development of the hyphal length and tip densities. The model parameters include the tip elongation rate v , the hyphal death rate d , the tip death rate d_n , the tip branching rate b_n , the maximal tip density n_{\max} , the tip-hypha anastomosis rate a_1 , the tip-tip anastomosis rate a_2 , the proliferation parameter for the hyphal tip density at the root-soil boundary a , and the initial tip density at the root-soil boundary $n_{0,b}$. No direct measurements of these growth parameters were available. Therefore, our aim was to fit the fungal growth model to the measured hyphal length densities in order to obtain the set of parameters which gave the best fit. Computing the respective dimensionless parameters (Schnepf *et al.*, 2008) allowed us to interpret different growth patterns for the different fungal species. The dimensionless parameter δ describes the relative importance of hyphal death with respect to apical branching. The relative importance of nonlinearity in apical branching and tip-tip anastomosis with respect to elongation is described by the dimensionless parameter β ; α describes the relative importance of tip-hypha anastomosis with respect to branching and ξ and ν are the dimensionless parameters for the boundary conditions.

The data on hyphal length density were not collected primarily for model validation. This resulted in the fact that the number of data points was low with respect to model parameters and also that only data points for one harvest point were available. Therefore, we reduced the complexity of the model as much as possible by trying to fit the simplest version of the model first and including processes (anastomosis and boundary proliferation) only if the model fit could be increased.

Following assumptions and preparatory steps were made before the model could be run:

1) HLD in the substrate with non-mycorrhizal plants was subtracted from the HLD of each species; units were converted from m/g to m/cm³ by using the measured bulk density of 1.3 g ml⁻¹.

2) Each mean was calculated from 5 replicates and the standard error of the mean (ste) obtained as std/\sqrt{n} (with std = standard deviation and n = number of replicates, here 5)

3) Due to subtracting of HLD in the substrate with non-mycorrhizal plants from HLD of each mycorrhizal species, both those datasets contributed to the overall error ste_T for each point. It was calculated according to the Gaussian law of error propagation (Walpole *et al.*, 2007):

$$ste_T = \sqrt{ste_{myc}^2 + ste_{NM}^2} . \quad (\text{Equation 3})$$

(with ste_{myc} = standard error calculated for the AMF species considered and ste_{NM} = standard error calculated for the control treatment)

4) Simulation time: 42 days

5) The HLD inside the root compartment was taken to be the boundary condition for the hyphal compartment

The model was compared to measured data by computing the root mean squared error (RMSE) (Smith *et al.*, 1997):

$$RMSE = \frac{100}{\bar{O}} \sqrt{\frac{\sum_{i=1}^n (P_i - O_i)^2}{n}} \quad (\text{Equation 4})$$

where \bar{O} is the mean of the observed data, O_i are the i measurements of hyphal length density, n is the number of data points and P_i are the i model predictions. The statistical significance of RMSE was assessed by comparing to the 95% RMSE confidence interval (Smith *et al.*, 1997):

$$RMSE_{95\%} = \frac{100}{\bar{O}} \sqrt{\frac{\sum_{i=1}^n (t_{(n-2)95\%} - ste_T(i))^2}{n}} \quad (\text{Equation 5})$$

When $RMSE < RMSE_{95\%}$, then the model is acceptable.

Results

Inocula of all three fungi were highly infective. On average, colonization by *Glomus intraradices* reached 85% root length, followed by *Gigaspora margarita* (54%) and *Glomus claroideum* (41%). Colonization of roots by arbuscules were following the same trend, reaching on average 40% of the root length. Vesicles were only recorded in roots of medic inoculated with either of the two *Glomus* species. No mycorrhizal colonization structures were observed in the non-mycorrhizal (NM) control roots.

Mycorrhizal inoculation resulted in a significant promotion of the plant biomass and of the P uptake (Table 1; Fig. 3). The two *Glomus* treatments were more beneficial for the plant growth compared to the *Gigaspora* and the NM treatments (Table 1). Plant P uptake was also promoted by the three different mycorrhizal treatments (Table 1), with greatest benefits conferred by *G. intraradices*, followed by *G. claroideum* and by the *Gigaspora*. Those differences resulted in the P use efficiency being smaller for the *G. intraradices* treatment as compared to all other treatments (Table 1).

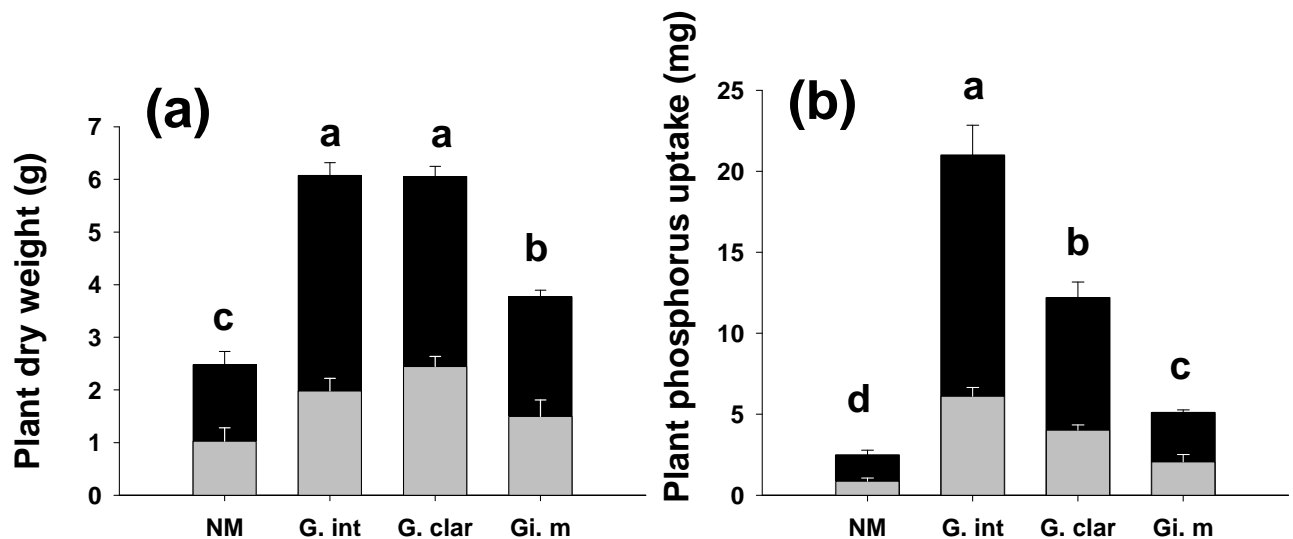


Figure 3

Combined shoot (black) and root (grey) biomass (a) and phosphorus uptake (b) of medic plants per pot at harvest. Mean values of five independent replicates \pm standard deviations of the means are shown. Different letters indicate significant differences between the means according to least significant difference-based multiple range test following significant ANOVA ($p < 0.05$).

Table 1: Mycorrhizal plant growth and phosphorus uptake benefits (%) and phosphorus use efficiency of medic plants being colonized by the different AMF species. Mean values of 5 replicates accompanied by standard deviations of means are shown. Different letters indicate significant differences among the means ($p < 0.05$)

	Plant growth benefit (%)	Phosphorus uptake benefit (%)	Phosphorus use efficiency (g mg⁻¹)
NM	0 ± 18 c	0 ± 14 d	1.00 ± 0.100 a
<i>G. intraradices</i>	145 ± 16 a	745 ± 77 a	0.30 ± 0.008 d
<i>G. claroideum</i>	144 ± 14 a	390 ± 33 b	0.50 ± 0.050 c
<i>Gi. margarita</i>	52 ± 10 b	105 ± 14 c	0.74 ± 0.040 b
ANOVA	F(3, 16) = 117.25***	F(3, 16) = 289.02***	F(3, 16) = 120.4***

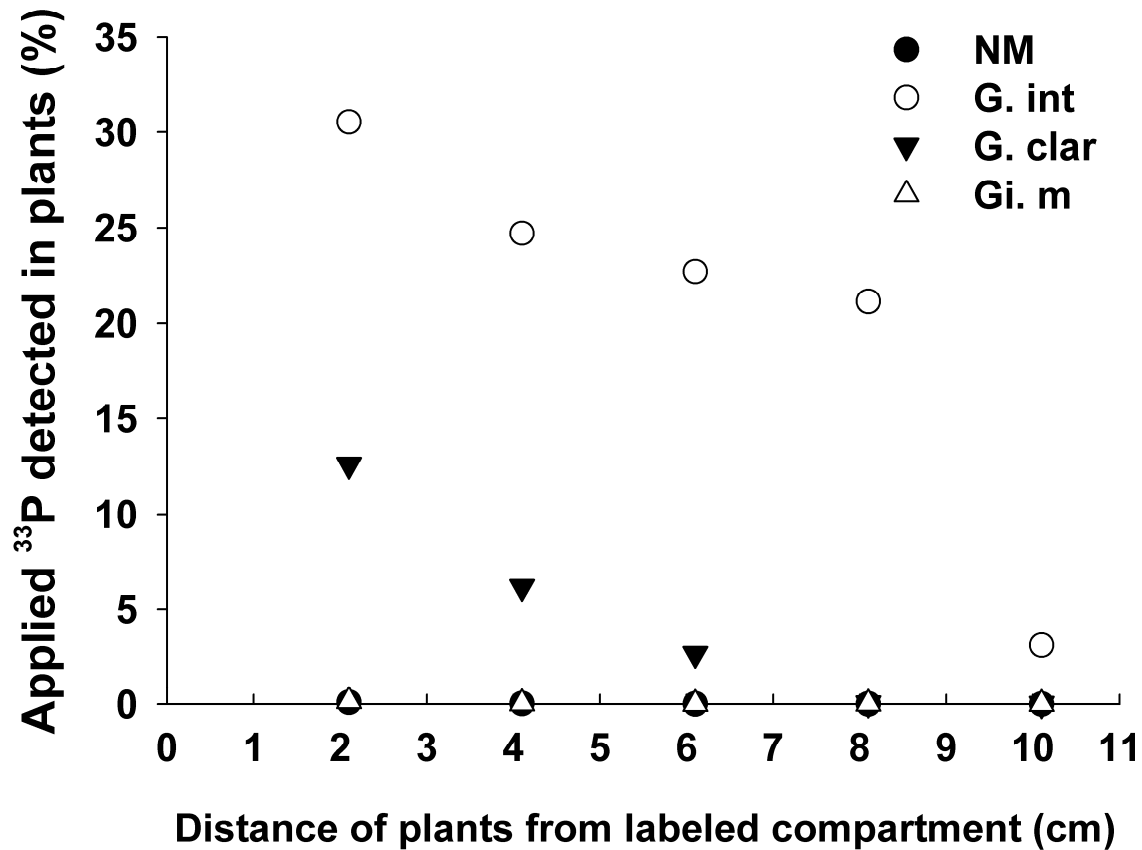
*** $p < 0.001$

Appearance of radioactivity in plant shoot measured by hand monitor during the experiment (Table 2) showed faster elongation rate of the mycelium front for *Glomus intraradices* with 0.5 cm day⁻¹ compared to 0.2 and 0.03 cm day⁻¹ for *Glomus claroideum* and *Gigaspora margarita*, respectively.

Further, the radioactivity of ³³P found in the medic plants (shoot and root combined) gives an indication of the maximal distance reached by the mycorrhizal hyphae when growing into the root-free zone (Fig. 4) within the duration of the experiment. The percentage of ³³P transported to the NM plants from the labeled compartment nearest to the 30 µm mesh was close to zero (0.09 % of the applied amount). In plants colonized by *Glomus intraradices*, significant amounts of ³³P were transferred to the plants via the mycelium even from the greatest distance, 9.1 cm from the roots (Fig. 4). For *Glomus claroideum*, the ³³P uptake took place at a maximal distance of 5.1 cm, while almost no ³³P activity was detected in any of the plants colonized by *Gigaspora margarita* (Fig. 4).

Table 2: Appearance of radioactivity in plant shoot as recorded by a hand-held monitor (Bq cm⁻²). The radioactivity was measured 24, 27, 31, 34 and 38 days after sowing by a UMo monitor (EG & G Berthold, Bald Wildbald Germany) by placing the detector surface above the plant compartment as shown in Figure 2 to avoid potential interference with radioactivity emitted from the labeled compartment. Single values are shown for each distance of ³³P placement and each fungal treatment. NM – non-mycorrhizal control, G. int – *Glomus intraradices*, G. clar – *Glomus claroideum*, Gi. m – *Gigaspora margarita*. Numbers accompanying treatment designation indicate distance of plants from the ³³P labeled compartment. These measurements enabled the calculation of the elongation rate of the mycelium front of the AMF species by considering on average the time necessary for the mycelium to come across the distances between the different labeled compartments (elongation rate = distance.time⁻¹)

Treatments	24 d	27 d	31 d	34 d	38 d
NM-2.1	0.1	0.2	0.2	0.2	0.2
NM-4.1	0.1	0.2	0.2	0.1	0.2
NM-6.1	0.1	0.2	0.2	0.2	0.2
NM-8.1	0.2	0.2	0.2	0.1	0.2
NM-10.1	0.1	0.2	0.2	0.2	0.2
G. int - 2.1	5.0	15.0	30.0	41.0	33.5
G. int - 4.1	0.2	1.7	6.2	15.3	20.8
G. int - 6.1	0.1	0.4	1.9	6.9	15.8
G. int - 8.1	0.2	0.2	0.7	5.1	12.7
G. int - 10.1	0.1	0.2	0.2	0.1	1.2
G. clar - 2.1	1.2	7.0	11.2	14.3	17.5
G. clar - 4.1	0.1	0.2	0.9	4.3	8.3
G. clar - 6.1	0.2	0.2	0.2	0.3	1.3
G. clar - 8.1	0.1	0.2	0.2	0.2	0.1
G. clar - 10.1	0.2	0.2	0.2	0.2	0.1
Gi. m - 2.1	0.1	0.1	0.1	0.2	0.3
Gi. m - 4.1	0.1	0.2	0.1	0.2	0.1
Gi. m - 6.1	0.2	0.1	0.2	0.1	0.2
Gi. m - 8.1	0.1	0.2	0.2	0.2	0.1
Gi. m - 10.1	0.1	0.2	0.2	0.2	0.2

**Figure 4**

Percentages of ³³P applied into the root-free zone and detected in the plants at harvest (shoot and roots combined). Single values are shown for each distance of ³³P placement and each fungal treatment. NM – non-mycorrhizal control, G. int – *Glomus intraradices*, G. clar – *Glomus claroideum*, Gi. m – *Gigaspora margarita*.

The HLD was low and almost identical for the NM treatment at all sampling positions (gross average of $0.3 \pm 0.12 \text{ m g}^{-1}$), with no significant differences between sampling positions ($p = 0.20$; Fig. 5). For plants being colonized by *Glomus intraradices*, the HLD measured was significantly different from the NM treatment for all sampling position ($p < 0.001$) and significantly varied along the cuvette ($p < 0.001$) with a maximum of $7.7 \pm 1.3 \text{ m g}^{-1}$ at a distance of 2.1 cm from the 30 μm mesh. For the *Glomus claroideum* treatment, the maximal hyphal density was measured in the plant compartment ($5.95 \pm 1.4 \text{ m g}^{-1}$) which was not statistically different from the HLD measured at 2.1 cm from the mesh ($4.56 \pm 2.9 \text{ m g}^{-1}$). In the last compartment (at a distance of 10.1 cm) the HLD of *Glomus claroideum* was not significantly different from the NM treatment. In the case

of *Gigaspora margarita* treatment, the HLD measured across different distances from the plants showed a sharp decrease from the plant compartment, where almost 23 m g^{-1} were recorded, towards to root-free zone (Fig. 5). Already at a distance of 2.1 cm from the roots, the measured HLD values were not significantly different from the NM treatment.

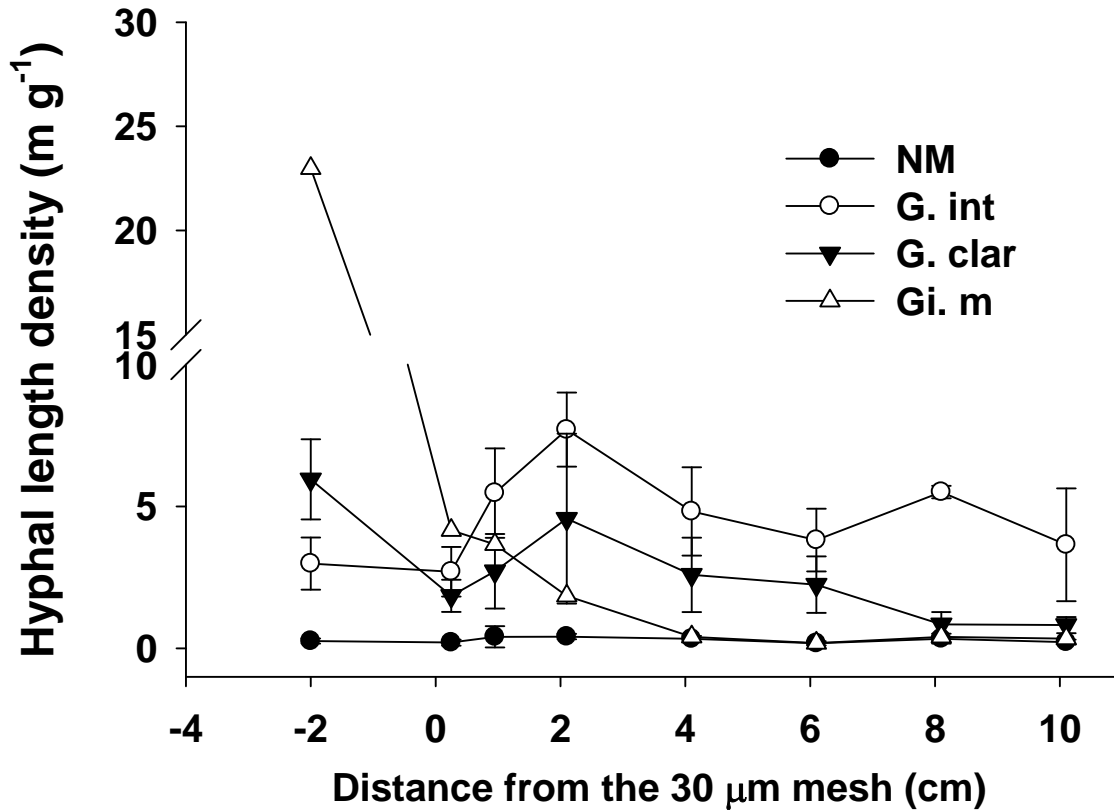


Figure 5

Mycelium length density in the plant compartment (negative distance from the mesh) and in the root-free zone at increasing distances from the 30 µm mesh. Values are given per unit of dry weight of the substrate. Mean values of five independent replicates \pm standard deviations of the means are shown. NM - non-mycorrhizal control, G. int – *Glomus intraradices*, G. clar – *Glomus claroideum*, Gi. m – *Gigaspora margarita*.

Transfer of ^{32}P was significantly different between the three mycorrhizal treatments (Fig 6a, $p < 0.001$). Almost no ^{32}P was found in the NM plants (0.08% of the applied amount). The mycorrhizal plant P uptake pathway efficiency were different between the different AMF species ($p = 0.01$). The values reached 3.17 ; 2.94 and $0.38 \text{ kBq } ^{32}\text{P} (\text{m g}^{-1})^{-1}$ of HLD for the *Glomus intraradices*, *Glomus claroideum* and *Gigaspora margarita* treatments, respectively. The two *Glomus* species created a statistically homogeneous group with

comparable P efficiencies, whereas *Gigaspora* appeared to have significantly lower mycorrhizal P uptake efficiency. The percentage of ^{32}P used for the labeling and found in the extracted AMF mycelium from the buffer zone 1 was almost six times higher in the *Gigaspora* mycelium than in that of the two *Glomus* species (Fig. 6b).

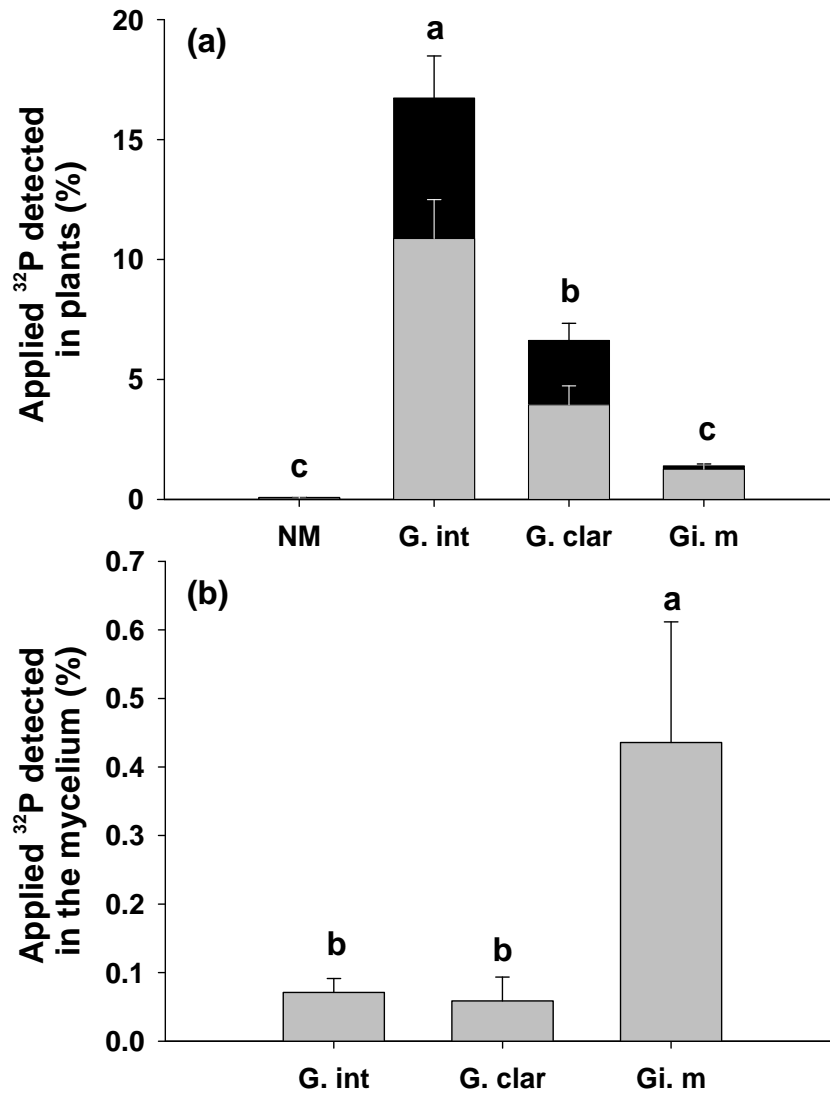


Figure 6

Percentages of ^{32}P applied into the root-free zone and detected in the plants (a) at harvest (shoot and roots combined), and in the mycelium (b) extracted from the buffer zone 1, 48 h after labeling. Mean values of five independent replicates \pm standard deviations of the means are shown. Different letters indicate significant differences between means according to least significant difference-based multiple range test following significant ANOVA ($p < 0.05$). NM - non-mycorrhizal control, G. int – *Glomus intraradices*, G. clar – *Glomus claroideum*, Gi. m – *Gigaspora margarita*.

The numerical model of AMF hyphal growth (Schnepf *et al.*, 2008) was fitted to the hyphal length densities of the three fungal species, starting with the most reduced form of the model (linear branching only and no anastomoses) and adding those additional mechanisms only as required. This procedure ensured that the number of model parameters was as small as possible and would not exceed the number of data points available. RMSE was used as a measure for the model fit. Generally, the lower the RMSE, the better is the model fit. To make the choice of which value is acceptable less subjective, we followed Smith *et al.* (1997) and compare the RMSE with the RMSE_{95%}. The results showed that the model could be fitted to all the fungal species within the accuracy of the data provided (Fig. 7). The best fit was obtained for the species *Gigaspora margarita*, where the fitted curve literally passes through all the data points, while the fitted curves miss several data points of the other two species. The respective model parameter values as obtained from the fitting are shown in Table 3.

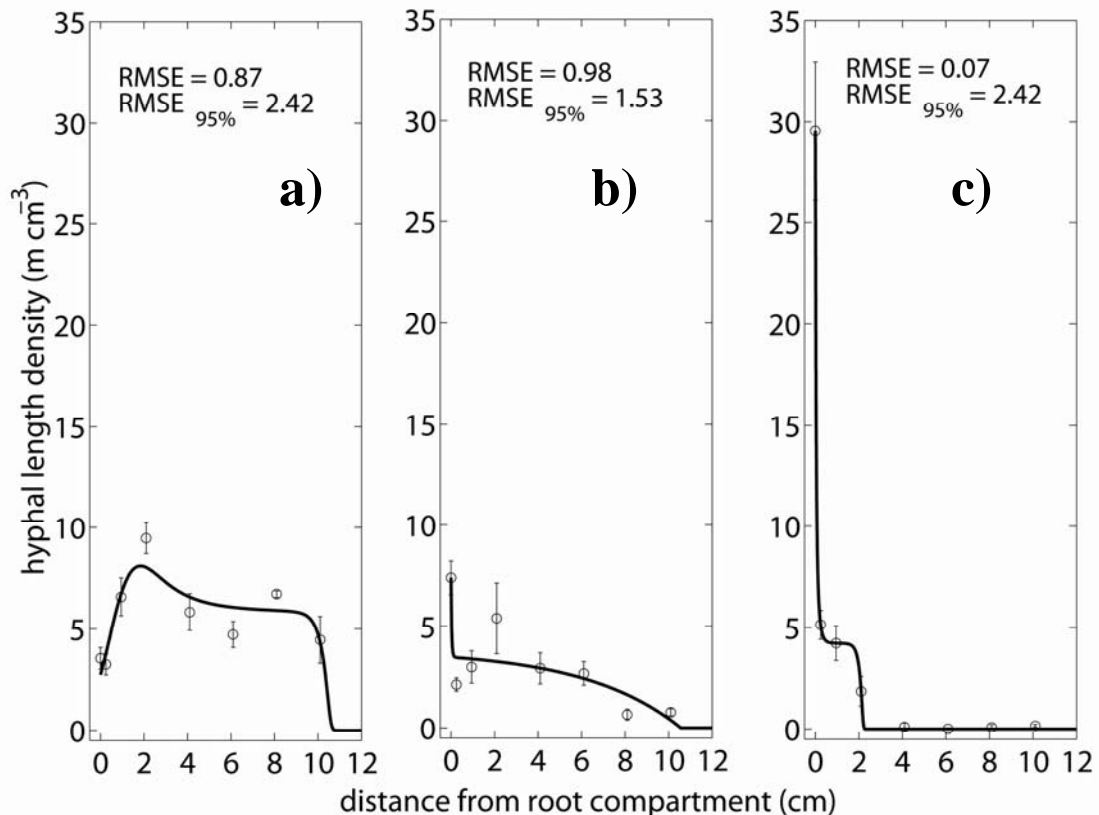


Figure 7

Fitting the hyphal length densities in the substrate to the growth model for the AMF mycelium. (a) *G. int*, number of model parameters: 7 (b) *G. clar*, number of model parameters: 6 (c) *Gig. m.*, number of model parameters: 6.

Table 3: Parameter values for hyphal growth model

Symbol	Name	Units	<i>G. int</i>	<i>G. clar</i>	<i>Gi. m</i>
v	elongation rate	cm s^{-1}	0.22960	0.20082	0.04366
b_n	apical branching rate	s^{-1}	0.7642	8.9228	0.4714
d_n	tip death rate	s^{-1}	0.2770	0.0564	0.0843
d	hyphal death rate	s^{-1}	0.0006	0.0566	0.3643
n_{max}	inverse of the maximal tip density	cm^{-3}	2744.6	108.7	4291.8
$a_{n\rho}$	tip-side anastomosis rate	$\text{cm}^3 \text{cm}^{-1} \text{s}^{-1}$	0.0981	-	-
a	root-soil boundary proliferation rate	$\text{cm cm}^{-3} \text{s}^{-1}$	0.0656	-	-
$\rho_{0,b}$	initial tip density at root-soil boundary	cm^{-3}	-	8.1573	29.5434

The dimensionless parameters in Table 4 show the relative importance of the different growth mechanism for each fungal species. These results indicate that:

- 1) The important mechanisms for *Glomus intraradices* are both nonlinear branching and tip-hyphal anastomoses. This implies that this species scavenges locally for resources and creates an interconnected mycelium for facilitated resource transport within the mycelium.
- 2) *Glomus claroideum* mycelium network growth is strongly dominated by nonlinearity in apical branching, implying that this species reaches the maximal tip density very quickly and is very efficient in local scavenging for resources.
- 3) The important mechanisms for *Gigaspora margarita* are nonlinear branching, but also hyphal death, and a high tip density at the root compartment (i.e. root fungus interface is important for this species).

Tip proliferation at the root fungus interface was not important for any of the fungal species, implying that the mycelium inside the root compartment was well established at the time of the experiment harvest.

Table 4: Dimensionless parameter values for hyphal growth model

Symbol	Name	<i>G. int</i>	<i>G. clar</i>	<i>Gi. m</i>
δ	relative importance of hyphal death with respect to apical branching	0.0012	0.0064	0.9412
β	relative importance of nonlinearity in apical branching and tip-tip anastomosis in relation to elongation	1.1487	302.5608	7.4319
α	relative importance of tip-hypha anastomosis with respect to branching	1.9073	-	-
ξ	dimensionless proliferation at boundary	$8.5 \cdot 10^{-6}$	-	-
ν	dimensionless initial tip density at boundary	0.0142	0.0070	0.9412

Discussion:***Root colonization extent:***

Reasonable levels of root colonization (40% minimum) were observed for the three AMF treatments, whereas no colonization was observed in the NM treatment. These colonization levels are rather high compared to some previously published studies (Li *et al.*, 2008). Although we admit that there is no general consensus about how much colonization is the best for maximizing benefits of a particular plant-AMF combination, it appears that some arbitrary criteria to achieve comparability of different inoculation treatments is necessary (Abbott *et al.*, 1992). This is especially important if fungal traits like development of ERM, the support of plant growth and plant P uptake responses to inoculation are compared (Abbott & Robson, 1985), since these will depend on the root colonization levels. If root colonization levels would not be taken care of, different colonization levels may introduce a great bias in interpretation of the results.

Plant responses:

All mycorrhizal treatments resulted in an improved plant biomass and phosphorus content as compared to the NM plants (Table 1), confirming the beneficial nature of all the fungi included in this experiment. The plant growth benefits were high for the two *Glomus* species (145 % on average) and somehow lower for *Gigaspora margarita* (52% on average). Other studies have reported similar plant growth benefits for *Glomus intraradices* and *Glomus claroideum* (Smith *et al.*, 2004; Jansa *et al.*, 2008) for *Medicago truncatula* colonized to a similar degree as in this study. In other studies, *Gigaspora*

species have been shown to provide limited symbiotic benefits, sometimes flipping over to negative benefits, i.e. plant growth depression (Burleigh *et al.*, 2002; Smith *et al.*, 2004; Li *et al.*, 2008).

The three mycorrhizal treatments resulted in a significantly improved P uptake when compared to the NM treatment. Unlike the plant growth response, the two *Glomus* species were significantly different in term of phosphorus uptake benefit with a value reaching 745% on average for *Glomus intraradices* and 390% for *Glomus claroideum*. These are high values, comparable with the P benefits in low P soils (e.g. Jakobsen *et al.* (1992a)), but can also be explained by access of the fungi into soil volume which were not accessible to the NM roots due to spatial confinement. This may lead to overestimation of mycorrhizal benefits as discussed by Smith *et al.* (2000).

P mycorrhizal uptake pathway and ability of AMF to colonize the substrate

Important differences in P acquisition strategies were observed between the three mycorrhizal fungi. First, the ^{33}P activity detected in mycorrhized plants gives a good approximation of the extent of substrate colonization by the three different fungi, probably even better than the laborious measurement of the HLD, which does not allow distinguishing living and dead hyphae as well as does not allow counting AMF mycelium separately from other soil saprophytic/parasitic fungi. The maximal distances reached by the mycorrhizal hyphae from the roots were 10, 6 and 1 cm for *Glomus intraradices*, *Glomus claroideum* and *Gigaspora margarita* respectively. The results of this experiment for this particular point for the two *Glomus* species confirmed those obtained by a previous study (Jansa *et al.*, 2005) where a similar experimental design – though with a different host plant (maize). To our knowledge, the results presented here is the first time that a similar experiment is conducted with *Gigaspora margarita*. For a closely related *Gigaspora rosea*, congruent results were obtained, showing very limited expansion of hyphae into root-free zones (Smith *et al.*, 2003; Smith *et al.*, 2004). It is not clear, however, whether these results reflect a real feature (limited expansion capacity of the mycelium away from the roots) or whether the meshes present a physical barrier for (generally thick) *Gigaspora* hyphae (diameter estimated by microscopy: 8 μm on average versus 4-5 μm for the *Glomus* species).

In order to further characterize the implication of the mycorrhizal hyphae development in the P acquisition, the hyphal length density (HLD in m per gram of dry substrate) has been measured for the three species in the different compartments of the root-free zone and in the plant compartment. The results support the ^{33}P data's with the exception of *Glomus claroideum*, whose mycelium was observed and quantified at 8.1 cm from the root. This could be explained by a small delay of the ^{33}P transfer to the plants for a mycelium that had probably just started to colonize this particular labeled compartment – or by generally limited capacity of this species to transport P over greater distances. In the plant compartment, the treatment with *Gigaspora margarita* has produced large amounts of mycelium with a value reaching on average 23 m of hyphae per gram of dry substrate. This result is consistent with many other studies (Hart & Reader, 2002c) that have shown that members of this genera usually colonize intensively the substrate close to the roots but no specific distance had ever been mentioned.

Knowing the HLD for the different compartments, it is possible to calculate how many meters of hyphae have been produced on average by each mycorrhizal treatment for the whole cuvette system. *Gigaspora margarita* is first with an average total of 3×10^4 m of hyphae produced, then *Glomus intraradices* and *Glomus claroideum* with 2×10^4 m and 1.45×10^4 m respectively. These numbers indicate that it is more the maximal distance reached the hyphae than the total mycelium production that explains the diversity observed in the phosphorus acquisition of mycorrhized plants.

Modeling of HLD indicates important differences between the three AMF used in this study. It is the first study comparing two species of the same genus *Glomus*, which means that important differences in mycelium growth patterns also exist between the different species of the same genus. Precautions should be taken for the output of this modeling because, first, it has been established with data collected at one point in time only and, second, with a number of data inferior to the number of full model parameters (8 versus 11). Future study will need sequential harvests in time and some direct measurements of hyphal turnover/vitality. Moreover, the general assumptions of the model (all the hyphae have the same diameter, the same growth rate and are considered either living or dead only) result in important simplifications of the reality. Nevertheless, the outputs of the model (dimensionless parameters) somehow reflect the different strategies of the AMF

for P acquisition, with for instance *Glomus intraradices*, whose hyphal length density is higher in the root-free zone than in the plant compartment. Like in other studies (Jakobsen *et al.*, 1992a; Jansa *et al.*, 2003a), this ability of *Glomus intraradices* to produce more mycelium at a distance from the roots would explain why this fungus demonstrates a better ability to explore the substrate and to provide the highest P uptake benefits. Care should be, however, exercised when extrapolating the differences identified between the different AMF isolates to differences between species – more work would be necessary to substantiate this as well as the species concept of the AMF should have been improved before. Further, the modeling part of this study indicates important mechanistic differences in mycelium expansion and probably in hyphal turnover between the studied AMF. It needs to be yet proven whether the mechanisms identified as key differences between the different AMF in the model are correctly assigned or whether mechanisms like anastomoses might have pointed to differences in other mechanisms such as proliferation of thin hyphal structures.

Efficiency of the mycorrhizal P uptake pathway

Six weeks after the start of the experiment, it was assumed that all the three mycorrhizal treatments would have produced extraradical mycelium at least in a small distance from the roots. The ^{32}P labeling was therefore employed to assess the efficiency of the mycorrhizal plant P uptake pathway. The activity found in the plants indicates an important diversity in this P uptake efficiency while the HLD in the labeled compartment were in the same range for the three species. The different values of the ^{32}P transfer by the different AMF to the plants (3.17; 2.94 and 0.38 kBq. of ^{32}P transferred to the plants (m g^{-1})⁻¹ of HLD for respectively the *Glomus intraradices*, *Glomus claroideum* and *Gigaspora margarita* treatments) seem to indicate that the HLD and the P uptake via the mycorrhizal pathway may be well correlated for closely related species but that they may be very different for phylogenetically distant species. These inter-generic differences have been observed in many studies (Smith *et al.*, 2004; Jansa *et al.*, 2005) and mainly concerned *Glomus* species. Studies comparing different isolates of the same species have shown that this correlation is even bigger within the species level (Munkvold *et al.*, 2004; Jansa *et al.*, 2005). Some ecological studies also suggest that the physiologic properties of closely related AMF species may be more similar than for the distant lineages, which

would also explain why plants usually establish mycorrhizal with different AMF species at the same time (Maherali & Klironomos, 2007)

Different reasons could explain the low ^{32}P activity detected in plants colonized by *Gigaspora margarita*. The density of phosphate transporters on the surface of the mycelium could be extremely low and/or the transporters poorly active (with a high K_m value). We can also speculate that more ^{32}P has been taken up but that its low activity in the plants is due to a delay of its delivery at the root-arbuscles interface. To test this last option, mycelium of the buffer zone 1 (see Fig. 1) has been harvested and further processed to determine its ^{32}P activity and phosphorus concentration in it at the time of harvest. The result presented in Figure 6b shows a significantly higher activity in the mycelium harvested in the *Gigaspora margarita* treatment. Similar picture was also observed with respect to the total P content of the extracted mycelium (data not shown). These results are in agreement with some previous studies (Jakobsen *et al.*, 1992b; Boddington & Dodd, 1998), where it has been suggested that AMF species from the Gigasporaceae might store their phosphate prior to transfer to the plant. This strategy may be interconnected with their life-cycle which is different from that of the *Glomus* species (Boddington & Dodd, 1999). Indeed, the time needed to complete the life-cycle of *Gigaspora*, up to formation of new spores, is longer than for most of the *Glomus* species, and *Gigaspora* spores are usually much larger than those of *Glomus* spp. The accumulation and retention of phosphate observed in this experiment might therefore be a mechanism used by *Gigaspora margarita* to regulate the phosphate transfer to the plant while maintaining (or even stimulating) sufficient carbohydrate transfer from the plant for the production of the important mycelium and for spore formation. This argument is supported by the ratios presented in Table 5. While biomass and phosphorus are distributed between roots and shoot in the same proportion for the three mycorrhizal treatments, the ratio for ^{32}P activity is significantly higher for *Gigaspora margarita*. It is probable that most of the ^{32}P activity that has been measured in the colonized root is actually contained in the intraradical hyphae. This phenomena has also been observed in a previous study where it was shown that a substantial proportion of phosphorus in roots colonized by *Gigaspora margarita* was indeed quantified in the fungal structures (Solaiman & Saito, 2001). The fact that the phosphate efflux and the decrease in poly-P

content in the hyphae were both enhanced by the addition of different forms of glucose supports the hypothesis outlined above.

Strategies of the mycorrhized plants for the P uptake

Results provided by this cuvette experiment can not tell in which proportion the phosphorus contained in the plant is taken up through the mycorrhizal pathway or through the direct root pathway. Nevertheless, the ^{33}P found in the mycorrhized plants indicates that in the case of the *Glomus* species, the phosphorus may come from far away while for *Gigaspora margarita* it clearly comes from a limited volume of substrate close to the roots. These differential spatial abilities to acquire P might help explain evidence that colonization by multiple AM fungal species can be more beneficial than colonization by one species (van der Heijden *et al.*, 1998b).

Table 5: Root-to-shoot ratios of dry weight (DW), phosphorus (P) content and ^{32}P activity and specific ^{32}P activity (for root and shoot combined) in medic plants being colonized by the different AMF species. Mean values of 5 replicates accompanied by standard deviations of means are shown. Different letters indicate significant differences among the means ($p < 0.05$).

	Root : shoot DW	Root : shoot P content	Root : shoot ^{32}P activity	Specific ^{32}P activity (kBq mg ⁻¹)
NM	0.71 ± 0.12 a	0.53 ± 0.11 ab	2.13 ± 0.68	0.03 ± 0.002
<i>G. intraradices</i>	0.48 ± 0.05 b	0.41 ± 0.06 b	1.96 ± 0.05 b	0.747 ± 0.096 a
<i>G. claroideum</i>	0.67 ± 0.05 b	0.50 ± 0.08 b	1.50 ± 0.23 b	0.506 ± 0.084 b
<i>Gi. margarita</i>	0.66 ± 0.17 b	0.69 ± 0.19 a	9.90 ± 2.50 a	0.264 ± 0.092 c
ANOVA	F(3, 16) = 4.06*	F(3, 16) = 4.30*	F(3, 16) = 45.6***	F(3, 16) = 76.25***

0.01 ≤ p < 0.05 ; *** p < 0.001

Carbon costs

The plant phosphorus use efficiency (PUE, Table 1) allows for indirect comparison of C costs of the plants associated with mycorrhizal symbiosis. It shows how many grams of plant biomass were built per unit of P taken up by the plant. The average values calculated for *Glomus intraradices* indicate that for the same quantity of phosphorus, the plant biomass produced is smaller than for any other mycorrhizal treatments. In other studies (Burleigh *et al.*, 2002; Jansa *et al.*, 2008), such a difference of the PUE for the two *Glomus* species has not been observed and indicates that in the case of our

experiment, the growth of the plants colonized by *Glomus intraradices* could have been limited either by the size of the cuvette and/or other limiting nutrients or by the carbon drain imposed by *Glomus intraradices* that has heavily colonized the roots.

This variability can be attributed to the different life-cycles of AMF that demonstrate diversity in their fungal biomass inside and outside the roots and possibly in their respiration rate (for the same biomass) (Hart & Reader, 2002c; Hart & Reader, 2002b; Hart & Reader, 2002a; Fitter, 2006). AM species such as *Gigaspora margarita* produces large spores and thick hyphae that in return will generate more CO₂ than species like *Glomus intraradices* or *Glomus claroideum*. This has been described in the study of Lendenmann *et al.* (in preparation). This work also gives explanations for the relatively low phosphorus use efficiency (PUE) of plants colonized by *Glomus intraradices* compared to plants colonized by *Glomus claroideum*. Indeed, the plants colonized by this latter took up on average less phosphorus (12.5 mg versus 21 mg for *Glomus intraradices*) but the plant biomass response was similar on average for the two treatments. With the results provided by the work of Lendenmann *et al.*, we can speculate that the difference is explained by a higher carbon cost of *Glomus intraradices* symbiosis which might be due to a higher degree of root colonization (85% versus 41% for *Glomus claroideum*) and/or to a higher intrinsic respiration rate. This will need further investigations that are discussed in the work of Lendenmann *et al.*

Conclusions:

Using the knowledge generated in the previous chapter, it was possible to establish medic plants colonized by different AMF species to a minimal degree of 40% of root colonization at a given time point and to 1) assess the contribution of morphological and physiological traits of the external mycorrhizal mycelium to the P acquisition and 2) to compare the mycorrhizal carbon costs associated with the established symbiosis.

Using dual radioisotope labeling we could show that the AMF species *Gigaspora margarita* was gathering P only from limited soil volume, establishing dense mycelium networks, and that the P taken up from the soil was probably immobilized in the fungal mycelium before being delivered to the plants. *Gigaspora margarita* also drained important amounts of carbon from the plants, and its colonization resulted in

consequence in negligible overall benefits to plant growth as compared to non-mycorrhizal control plants. The extent of soil colonization by the two different *Glomus* species was higher than for *Gigaspora margarita* and they provided differential amounts of P to the plants. However, since the *Glomus claroideum*, which proved less efficient in P acquisition than *G. intraradices* also required significantly less carbon from the plants than *Glomus intraradices*, the levels of growth promotion by both of the *Glomus* species were similar.

Finally and to summarize, the results provided by this experiment contribute to a better definition and understanding of the mycorrhizal parameters playing important roles in the functional diversity in regards to the P acquisition. These parameters are:

- Ability of AMF to colonize the substrate (and to a lesser extent the roots): rate and extent (maximal distance reached from the roots) of substrate colonization.
- Efficiency of the mycorrhizal plant P uptake pathway: efficiency of absorption of the inorganic phosphates (from the soil solution), transport (within the mycorrhizal mycelium) and delivery (at the root-arbuscles interface); processes probably influenced by the density and activity of the corresponding transporters and other enzymes involved.
- Carbon cost of the mycorrhizal symbiosis: requirements for carbohydrates fixed by the plant photosynthesis for growth and maintenance of the AMF.

The importance of these parameters for the functional diversity may be, in addition, modulated by the host plant species and environmental conditions – therefore, further research is necessary to address these interactions.

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Chapter 3: Nature of interactions between arbuscular mycorrhizal species colonizing medic root system: Competition or facilitation?

Abstract:

Arbuscular mycorrhizal fungal (AMF) communities were established in pots using fungal isolates from a single field in Switzerland. Their competitive ability when colonizing roots of medic (*Medicago truncatula*) were assessed in all possible two-species combinations using a range of inoculum densities. The root occupancy by the co-inoculated fungi was assessed using species-specific real-time PCR assays with TaqMan probes to determine the copy numbers of large ribosomal subunit genes of each AMF species. Nature of interactions between the AMF species was varying from competition to facilitation and was largely influenced by the identity of the co-inoculated fungi. No competitive exclusion of one of the AMF species co-inoculated with another species was observed in any combination of the AMF species in any inoculum density ratio. Facilitation was particularly strong if phylogenetically distant fungal species were combined. Medic plants co-inoculated with *Glomus claroideum* and *Glomus intraradices* acquired substantially more phosphorus than with any of the two *Glomus* species separately. This direct evidence of functional complementarity and the assessment of mycorrhizal interactions were greatly facilitated by use of real-time PCR.

Keywords:

Arbuscular mycorrhizal fungi (AMF), functional complementarity, *Gigaspora*, *Glomus*, growth and phosphorus uptake response, large ribosomal subunit (LSU), medic (*Medicago truncatula*); real-time PCR

Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that colonize roots of a wide range of host plants (Smith & Read, 2008). Apart from the well documented role of AMF in providing nutritional benefits, many other aspects of the symbiosis have been reported and include abiotic and biotic stresses resistance (Newsham *et al.*, 1995), plant community shaping (Hartnett & Wilson, 2002) and stabilization of soil aggregates (Rillig, 2004). There are numerous pieces of evidence that different AMF species and/or isolates largely differ with respect to their growth and physiology traits as well as with respect to the nutritional benefits conferred to their host plants (Jakobsen *et al.*, 1992a; Koch *et al.*, 2004; Munkvold *et al.*, 2004; van der Heijden *et al.*, 2004; Cavagnaro *et al.*, 2005; Hart & Reader, 2005; Jansa *et al.*, 2005; Koch *et al.*, 2006). This so called functional diversity that was also addressed in the previous chapter mainly results from diversity in 1) the ability of AMF to colonize the soil and (to a lesser extent) the roots, in 2) their efficiency to acquire, transport and deliver the nutrients to the host plant and in 3) the carbon cost of the established symbiosis (Grimoldi *et al.*, 2006; Lendenmann *et al.*, in preparation).

Almost all the data gathered on functional diversity of AMF have been obtained from experiments, where plants have been inoculated with single AMF isolates only. This is of course not relevant to the field situations, where it is well documented that AMF are present as a multi-species community in the roots and in the soil. The composition and diversity of such communities have been characterized within an abundant literature for both natural and agro-ecosystems and different physicochemical properties of soils (Jansa *et al.*, 2002; Jansa *et al.*, 2003b; Mathimaran *et al.*, 2005; Pivato *et al.*, 2007; Liang *et al.*, 2008; Rosendahl & Matzen, 2008).

Some of these studies have shown that the root system of individual plants is co-colonized by different AMF species (Daft & Hogarth, 1983; Van Tuinen *et al.*, 1998; Jansa *et al.*, 2003b). This observed co-occupation of the roots by different species is still poorly understood, and so is its meaning for the plant performance (functions fulfilled by the AMF community). Until now, only few studies have addressed whether such multiple colonizations are a result of competitive, synergistic or antagonist interactions (Alkan *et al.*, 2006; Jansa *et al.*, 2008). The lack of specific tools to detect and quantify different

AMF species in roots and/or in soils is one of the main reasons why such mechanisms are not yet well understood. Nevertheless, the recent introduction of quantitative real-time PCR technology has largely alleviated this problem and opened new avenues in research of AMF communities (Alkan *et al.*, 2004; Isayenkov *et al.*, 2004; Jansa *et al.*, 2008). With this technique, as it was described in the first chapter, a quantitative measure of the interactions among different AMF species should allow substantiation of the theory of functional complementarity that proposes that AMF species of complementary functions (e.g. uptake of nutrients from different soil pools) inoculated in mixtures may bring more benefits to the plant than any of the species separately (Koide, 2000).

In this context, the outcome of interactions occurring between different AMF species colonizing the same root system and its consequences for the host plant are closely linked. Indeed:

- i. For a particular AM fungus, the outcome of the interaction with other AMF species can be related to its infectivity potential (rate and extent of root colonization) and to its aggressiveness (ability of a fungus to maintain its level of colonization in a competitive situation (Wilson, 1984b)). Possible outcome for such interactions for this particular AM fungus is that its development will be either depressed (competition) or supported (facilitation) by the presence of another AMF in the roots and/or in the soil (no interaction at all is also possible but less likely).
- ii. As a consequence, outcomes of interactions occurring between AMF species will directly impact 1) the effectiveness of the mycorrhizal symbiosis due to changes in total colonization of root and soil and to mechanisms of functional complementarity as proposed by Jansa *et al.* (2008) and 2) the relative reproductive ability of the interacting fungi.

Literature addressing the questions of interactions has mainly focused on studies, where competitive abilities of different AMF isolates were compared (Daft & Hogarth, 1983; Wilson & Trinick, 1983; Abbott & Robson, 1984; Wilson, 1984b; Hepper *et al.*, 1988). The lack of isolate-specific tools enabling a quantification of the abundance in the root and in the soil of the interacting fungi has largely prevented major progress in that topic.

When different isolates of AMF are present in an inoculum, they will face several events of interactions that will result in a varying degree of root and soil colonization by each of the different AMF isolates. The time when the primary infection of the individual isolates occurs in the roots will result from a first event of competition for root penetration. The outcome of this competition for these individual AMF isolates will mainly depend on the density, distribution and state of activation of their infective propagules in the inoculum and in their rate of root interception. Data of Wilson (1984a) suggest that in subterranean clover, competition for penetration sites does exist and can occur even between different members of the same population. Inside the root, the different isolates will interact and most probably compete for space. Following standardization of the propagules density, infectivity becomes a measure of the ability of each fungus to spread within a root (Wilson, 1984a). It is mainly the characteristics of the secondary spread (to form secondary infections) of AMF that will give them a different competitive advantage during root colonization. By manipulating inoculum densities of different AMF in order to have similar rate of secondary infection formations in the root, some authors have proposed that fungi with high level of infectivity are less aggressive than other fungi that normally demonstrate low infectivity (Wilson, 1984b). Apart from competition, some studies have also outlined facilitation processes between AMF colonizing the same root system. This is the case of a study published by van Tuinen *et al.* (1998), where it has been shown that *Gigaspora rosea* and *Scutellospora castanea* occurred more frequently in the roots when in presence of other fungi. This work was done with semi-quantitative tools and more recent studies that have used real-time PCR have shown competition and/or facilitation, depending on the species of AMF considered in the co-inoculation, on the time of harvest and on the environmental conditions such as saline and phosphorus stresses (Alkan *et al.*, 2006; Jansa *et al.*, 2008). Interacting AMF may also compete for nutrients and (probably to a lesser extent) for water and oxygen (Clark, 1965). Other studies (Pearson *et al.*, 1993; Pearson *et al.*, 1994) suggested that access to sugars and the phosphorus status of the soils influenced the outcome of AMF interactions occurring in the same root system. The various carbon-sink imposed by the different AMF, whose strength depends on the identity of the fungus and on the plant species, as well as on environmental conditions, will result on a differential susceptibility of the root to further

colonizations by other AMF (Lerat *et al.*, 2003; Heinemeyer *et al.*, 2006). These mechanisms of competition have been observed within the same root-zone through possible mechanisms of plant-defense like reactions and between root fragments of different areas of the root system through possible mechanisms of signaling mediated by the host plant (Vierheilig, 2004b).

In this chapter, the main objective was to assess and characterize the nature of interactions occurring between different AMF species, when colonizing the same root system of medic plants. The consequences of these multiple colonizations for the host plant (in term of plant biomass and phosphorus uptake improvements) have been a secondary objective. To achieve these objectives, synthetic communities (assemblages consisting of two AMF species with varying inoculum densities) were established with three AMF species (*Glomus intraradices*, *Glomus claroideum* and *Gigaspora margarita*) isolated from a single field site in Switzerland (Jansa *et al.*, 2002). These fungi have been shown to differ in their strategies to acquire phosphorus from the soil (see chapter 2) and in their carbon cost (chapter 2, Lendenmann *et al.*, in prep). The quantification of AMF community composition in the roots, using the molecular assay described in the first chapter, have enabled assessment of the nature of interactions between the AMF species when colonizing the same root system of *Medicago truncatula*.

Material and methods

Experimental setup

The experiment was carried out in small plastic containers (80 ml each) arranged in plates 8 × 12 containers. These containers were filled with a substrate that has been homogeneously mixed with the mycorrhizal inoculum. Only containers bordering with other units from all 4 sides were used for growing experimental plants, edge rows were planted but not included in the experiment (to minimize border effect). The substrate consisted of sterilized field soil, coarse quartz sand (grain diameter 0.7- 1.2 mm) and fine quartz sand (grain diameter 0.08-0.2 mm) mixed in the ratio 1:3:1 (v:v:v). The soil was collected in Tänikon, Switzerland, air-dried, passed through 5 mm sieve and γ -irradiated at LEONI Studer Hard, Däniken, Switzerland, applying a dose of 25-75 kGy with ^{60}Co source. The properties of this substrate were described in chapter 1.

Experimental design

One host plant and three AMF species were considered. Non-mycorrhizal treatment was included in the design as well. The mycorrhizal treatments were separated into single species inoculations with five inoculum densities each, and double-species inoculations, where the inoculum density of one species was maintained constant and the density of the other (competitor) species varied similarly as for the single species inoculations (Fig. 1). This resulted into 43 different treatments, including the non-mycorrhizal (NM) control. Each treatment was replicated five times and additional containers with single species inoculations were prepared for time course assessment of the root colonization by the different mycorrhizal fungi.

Plants and AMF

Seeds of *Medicago truncatula* Gaertn. (medic) were surface-sterilized for 10 min in concentrated (97%) sulphuric acid (Massoumou *et al.*, 2007) and then washed with sterile water and germinated for 3 days on moistened filter paper. One seedling was planted into each container at the start of the experiment (here referred to as sowing time), and the harvest took place 42 days thereafter.

Three AMF species, all isolated from a single field site in Switzerland (Jansa, J. *et al.*, 2002), were used in this study. These were *Glomus intraradices* BEG 158, *Glomus claroideum* BEG 155 and *Gigaspora margarita* BEG 152. The inoculum was produced in 1 kg pots for 8 months and consisted of the same substrate as described above, planted with leek. The density of the AMF spores was assessed in each inoculum and then diluted with the potting substrate so as to reach comparable substrate infectivity (resulting in approximately 50% root length colonized by each fungus after six weeks of growth). These materials contained 40, 80 and 1200 spores per 80 ml volume for *Glomus intraradices*, *Glomus claroideum* and *Gigaspora margarita*, respectively, and were regarded as the 100% inoculum density of each species (level "1"). These inocula were then further diluted to reach a density equal to 40%, 10%, 2.5% and 1% of the level "1" (Fig 1). For the double inoculations, the inoculum density of one species was kept constant (corresponding to the level "1") and the inoculum density of the second (competitor) species was varied in the same pattern as for the single inoculations. Non-

mycorrhizal treatment was inoculated with substrate from pots, where non-mycorrhizal leek was grown for previous 8 months (5 g per container).

The growing plants were watered daily and received 15 ml plant⁻¹ week⁻¹ of a Hoagland nutrient solution (Hoagland & Arnon, 1950) containing no phosphorus throughout the duration of the experiment. The treatments were completely randomized in a growth chamber (Conviron PGV36, Winnipeg, Canada) under the following conditions: temperature 22/18°C and relative aerial humidity 75/90% (day/night, respectively), photoperiod 16h, combined fluorescent and incandescent light, 330 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Harvest and measurements

Plants were harvested 42 days after sowing. Extra pots for the time course assessment were harvested at 14 and 28 days after sowing. Upon harvest, plant shoots were cut and dried at 105°C for 48 h and weighed. Roots were washed from the substrate under tap water and then rinsed with deionized water, weighed, cut to about 1-cm pieces and mixed. Subsamples were taken from the single inoculation treatments for drying, DNA extraction, and root staining. From the dual inoculation treatments, subsamples of roots were taken for drying and DNA extraction only. Subsamples for drying were weighed before and after drying at 105°C for 48 h. The roots staining procedure was following the protocol described by Phillips and Hayman (1970) and Brundrett *et al.* (1984). Briefly, roots were macerated in 10% KOH (100g l⁻¹) at 90°C for 25 min, rinsed with water, incubated in 1% HCl (10g l⁻¹) at room temperature for 1 h, briefly rinsed with water before transfer to 0.05% Trypan blue (0.5g l⁻¹) in lactic acid: glycerol: water (1:1:1; v:v:v). In this solution, the roots were stained at 90°C for 2 h and then at room temperature overnight. Finally, the roots were de-stained for at least 24 h in water at room temperature. The extent of root length colonized by hyphae, arbuscules and vesicles was determined on stained roots according to the method of McGonigle *et al.* (1990), recording 50 intersections per sample. Dried root and shoot biomass samples were incinerated at 550°C for 4 h and ashes dissolved in 2 ml 65% HNO₃ (650g l⁻¹), made to 100 ml with distilled water and filtered through a paper filter (Whatman No 40). The concentration of P in the extracts was determined by flow injection analysis using colorimetric reaction after Boltz & Mellon (1948)

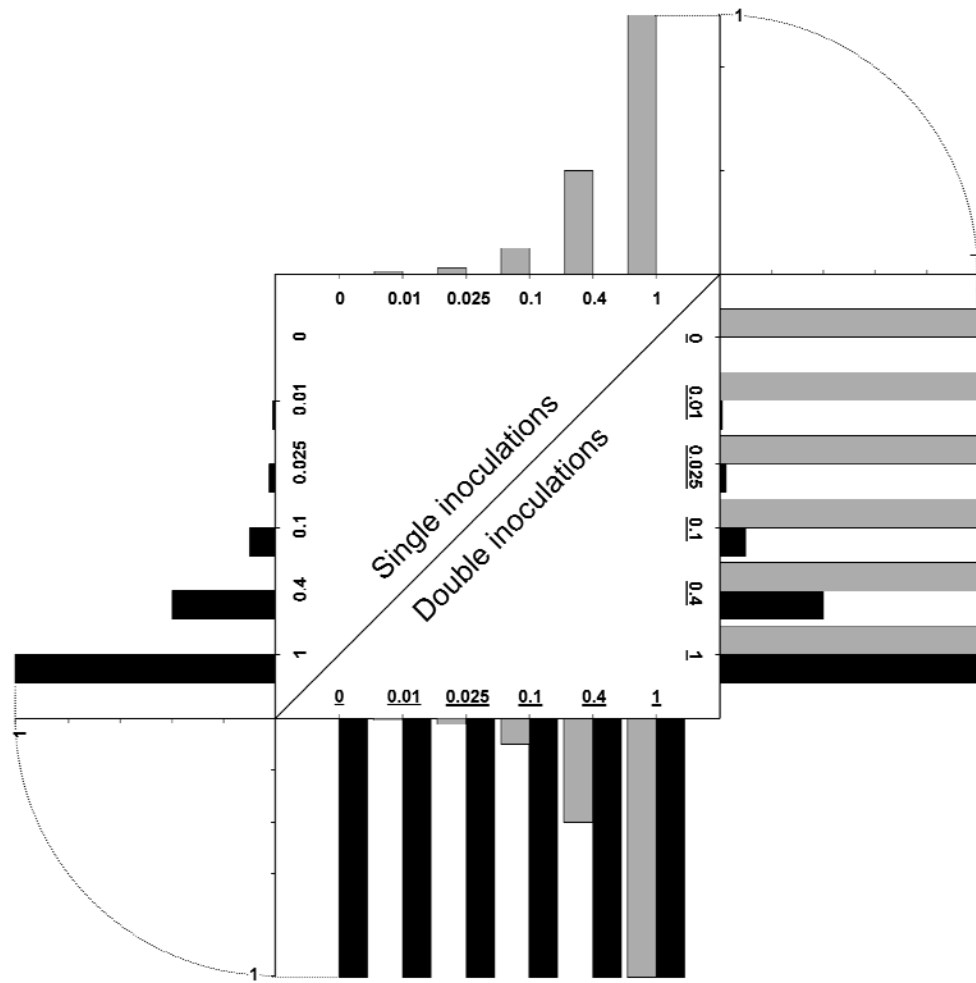


Figure 1: Experimental design of this study with single and double arbuscular mycorrhizal fungal (AMF) species inoculation treatments. For the single inoculations, five inoculation levels of each species have been considered (100%, 40%, 10%, 2.5%, and 1%). These levels are represented by fraction values in the figure (1 correspond to 100%, 0.4 to 40% and so on). The 100% inoculation level represents inoculum density of each species adjusted so that it results in approximately 50% root length colonized in 6 weeks after sowing (see Material and Methods for details). Black bars represent species 1 and grey bars represent species 2. For the double inoculations, the density of inoculum of one species was maintained at the level "1" (which corresponds to the 100% inoculum density) and the density of inoculum of the species 2 was varied in the same manner as for the single inoculations (underlined numbers). Considering the three AMF species included in this study, and all their possible combinations, experimental design of this study included a total of 42 mycorrhizal treatments (15 single inoculations and 27 double inoculations).

Quantification of mycorrhizal DNA by real-time PCR

The number of LSU copies of each AMF species was estimated in the roots by real-time PCR with species-specific primers and TaqMan probes as described in chapter 1. DNA was extracted by DNeasy Plant Mini Kit (Qiagen, Hombrechtikon, Switzerland) from the lyophilized root subsamples (100-150 mg fresh weight) after homogenization by bead disruption (Mini Bead Beater, BioSpec Products, Bartlesville OK, USA), following the manufacturer's recommendations. Before homogenization, all samples were spiked with a known number (5 billion copies) of an internal standard, which consisted of a cassava mosaic virus DNA fragment (GenBank accession AJ427910) carried in a pUC19 plasmid. Quantification of internal standard recovery in the DNA extracts, using a specific primer pair and a TaqMan probe, allowed for a correction for both DNA lost during the extraction and presence of unspecific PCR inhibitors in the DNA extracts (Jansa *et al.*, 2008). All the real-time PCR reactions were performed in the LightCycler 2.0 (Roche Diagnostics, Rotkreuz, Switzerland) using Roche chemistry with specific primers and probes labeled with fluorescein and BHQ-1 quencher as described in the chapter 1, using the DNA extracts diluted 5 times as template.

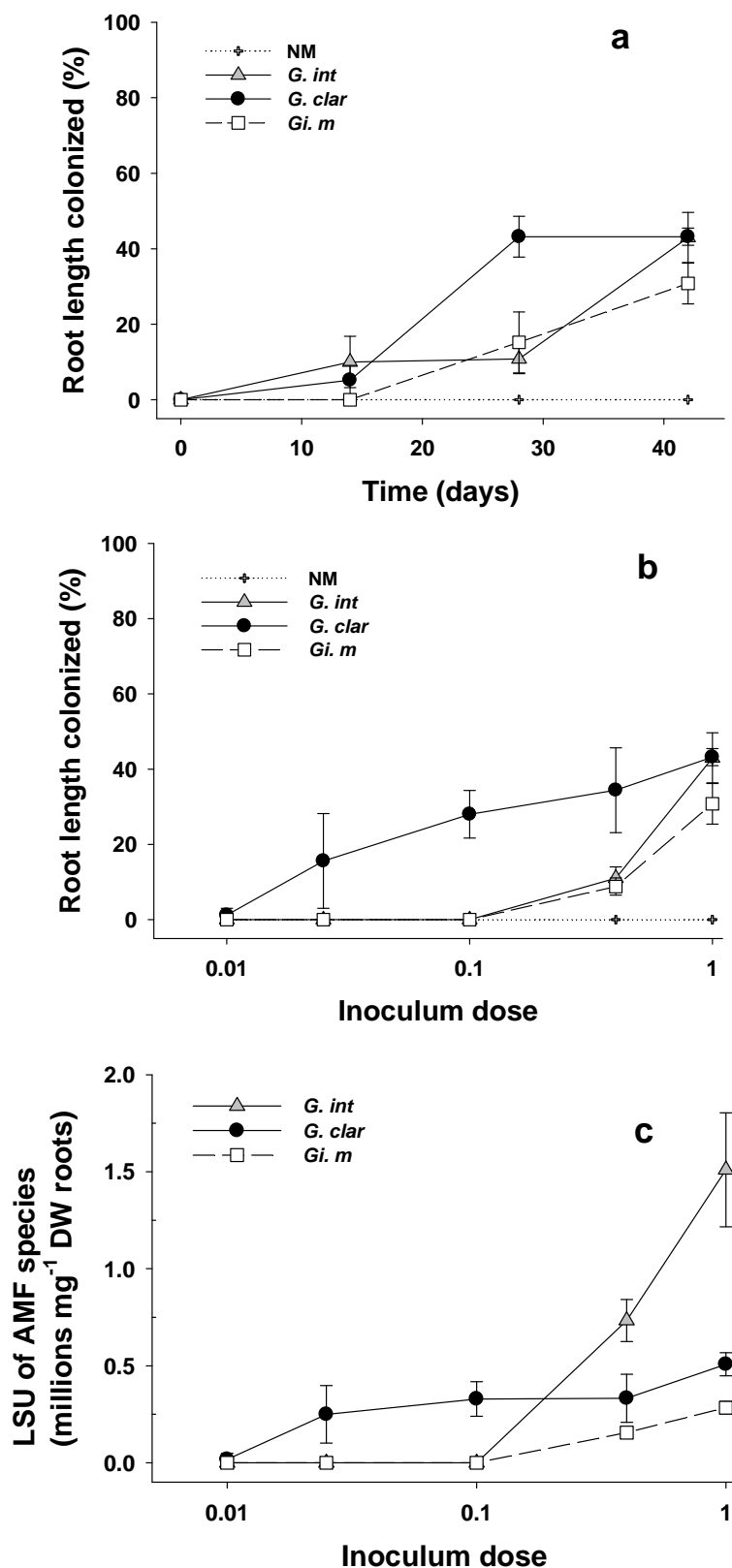
Calculation and statistics

The percentage of root length colonized by the AMF is given as the ratio of intersections containing any of the mycorrhizal structures to all observed root intersections per sample $\times 100$. Phosphorus concentrations in plant extracts were used for calculation of the plant P content. The LSU copy numbers per unit of root dry mass were calculated according to the equations presented in the chapter 1. The statistics (ANOVA, multiple range tests and *t*-test) were calculated in Statgraphics *Plus* for Windows version 3.1.

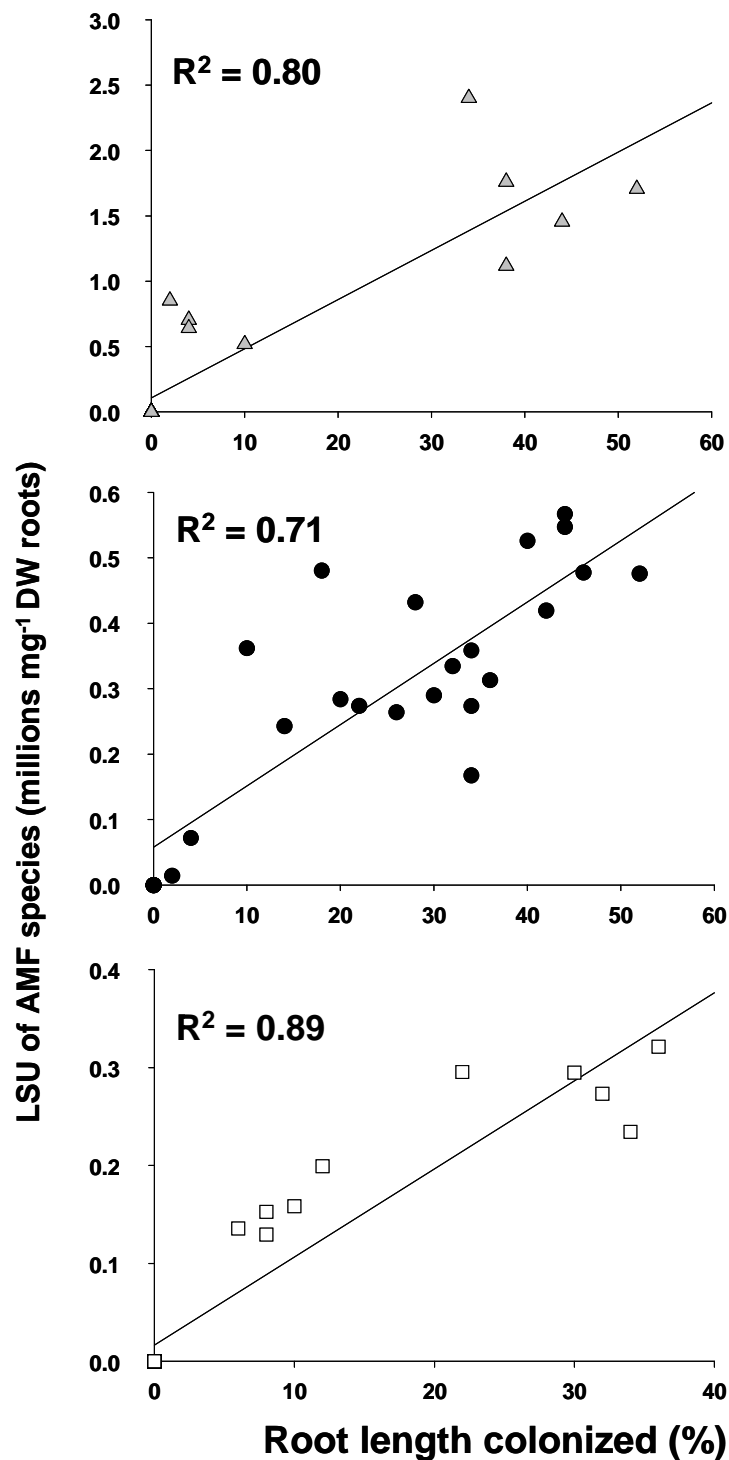
Results***Root colonization and its assessment***

Inocula of all three AMF species were infective when administered singly and in the highest dose (100% inoculum density), and the levels of root length colonization (RLC) were then similar among the different species 42 days after sowing, reaching on average 40% (Fig 2a). Data from the time course experiment indicated an earlier colonization of roots inoculated by *Glomus claroideum* as compared to the other two fungal species. No

mycorrhizal structures were observed in the non-mycorrhizal treatment at any time point (Fig. 2). Both staining and real-time PCR indicated that both *Glomus intraradices* and *Gigaspora margarita* only colonized roots when the inoculum was provided at 100% and 40% densities. If more diluted, the inoculation did not result in any detectable colonization (Figs. 2a and 2b). In contrast, detectable levels of colonization were measured for all inoculation doses of *G. claroideum* at 42 days after sowing (Figs. 2a and 2b), although the values for the most diluted inoculum (1% density) were very low. Numbers presented in Figure 2c show that for similar root length colonized by the different AMF species, the numbers of LSU copies per unit of root dry weight for *Glomus intraradices* is several times higher than for the other two AMF species. Highly significant correlations were found between the RLC and LSU copy numbers per root dry weight for the singly inoculated containers 42 days after sowing for all three AMF species (Fig. 3).

**Figure 2:**

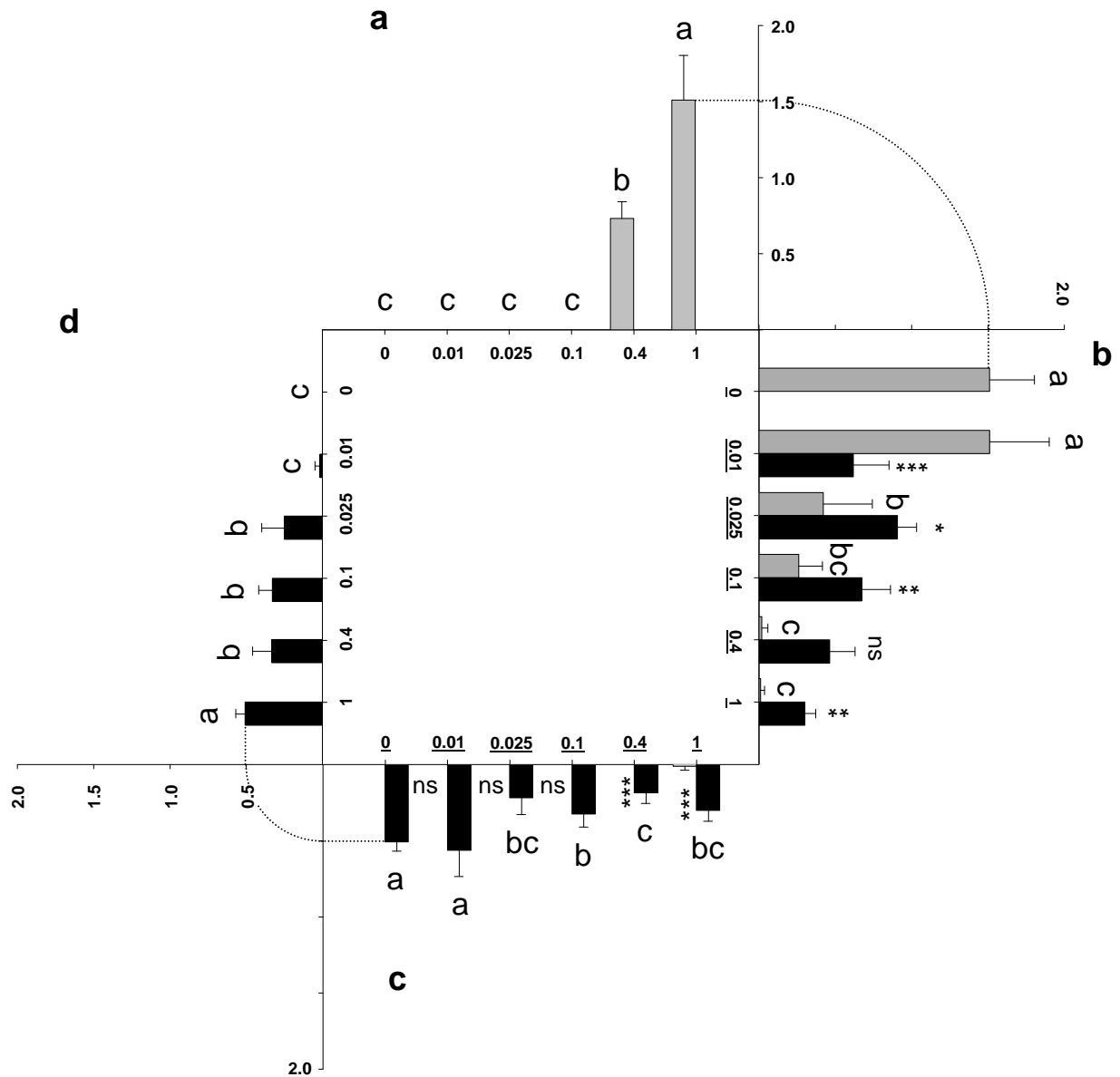
Development of colonization of medic roots by the three arbuscular mycorrhizal fungal species inoculated singly, as revealed by Trypan blue staining (a, b) and by real-time PCR with TaqMan probes (c). Results are shown for sequentially harvested plants, inoculated with 100% inoculum dose (level "1"; a), and for inoculum dilution series harvested 42 days after sowing (b, c). Mean values of five replicates \pm standard deviations are shown. NM – non-mycorrhizal treatment, *G. int* – *Glomus intraradices*, *G. clar* – *Glomus claroideum*, *Gi. m* – *Gigaspora margarita*, LSU – large ribosomal subunit copies, DW – dry weight.

**Figure 3**

Relationship between the proportion of root length colonized by the arbuscular mycorrhizal fungi and copies of the large ribosomal subunit (LSU) of the respective fungal species per unit of dry weight (DW) of roots. Root samples were obtained from plants inoculated by five different inoculum doses (5 replicates per dose), and all harvested 42 days after sowing. Triangles represent *Glomus intraradices*, circles *Glomus claroideum*, and squares *Gigaspora margarita*.

Interspecific competition

Plants co-inoculated with 100% inoculum density of *Glomus intraradices* and increasing inoculum density of *Glomus claroideum* showed a significant decrease of LSU copies number of *Glomus intraradices* (Fig 4b, grey bars). This decrease was correlated with the inoculum density of *Glomus claroideum* but not to its abundance detected in the roots (Fig 4b, black bars). When plants were co-inoculated with the same amount of the two inocula both at 100% density, the abundance of both species detected by the real-time PCR were significantly reduced compared to the respective single inoculation treatments. This reduction was much greater for *Glomus intraradices*, where only 0.7% was detected as compared to the respective single inoculation by *G. intraradices*, than for the *Glomus claroideum*, where 58% of the LSU copies were detected as compared to the single inoculation treatment. For lower inoculum densities of *Glomus claroideum* combined with the 100% density of *G. intraradices*, the LSU copy numbers of *Glomus intraradices* were reduced as noted before (except for 1% density by *Glomus claroideum*), but the LSU copy numbers of *Glomus claroideum* were mostly enhanced in comparison to the single inoculations with the respective inoculum densities (Fig. 4b and 4d, black bars). Plants inoculated with 100% inoculum density of *Glomus claroideum* and variable amounts of *G. intraradices* inoculum showed a significant reduction of LSU copy number of *Glomus claroideum* (Fig 4c, black bars) with increasing density of *G. intraradices* inoculum. However, this reduction (down to 37% of the single inoculation with *G. claroideum*, Fig. 4c) was far less pronounced than in the case of co-inoculation of 100% density of *G. intraradices* with variable densities of *G. claroideum* (Fig. 4b). Interestingly, this reduction of LSU copy numbers of *G. claroideum* was not correlated to the inoculum density of *Glomus intraradices* neither to its abundance in the roots (Fig. 4c, grey bars).

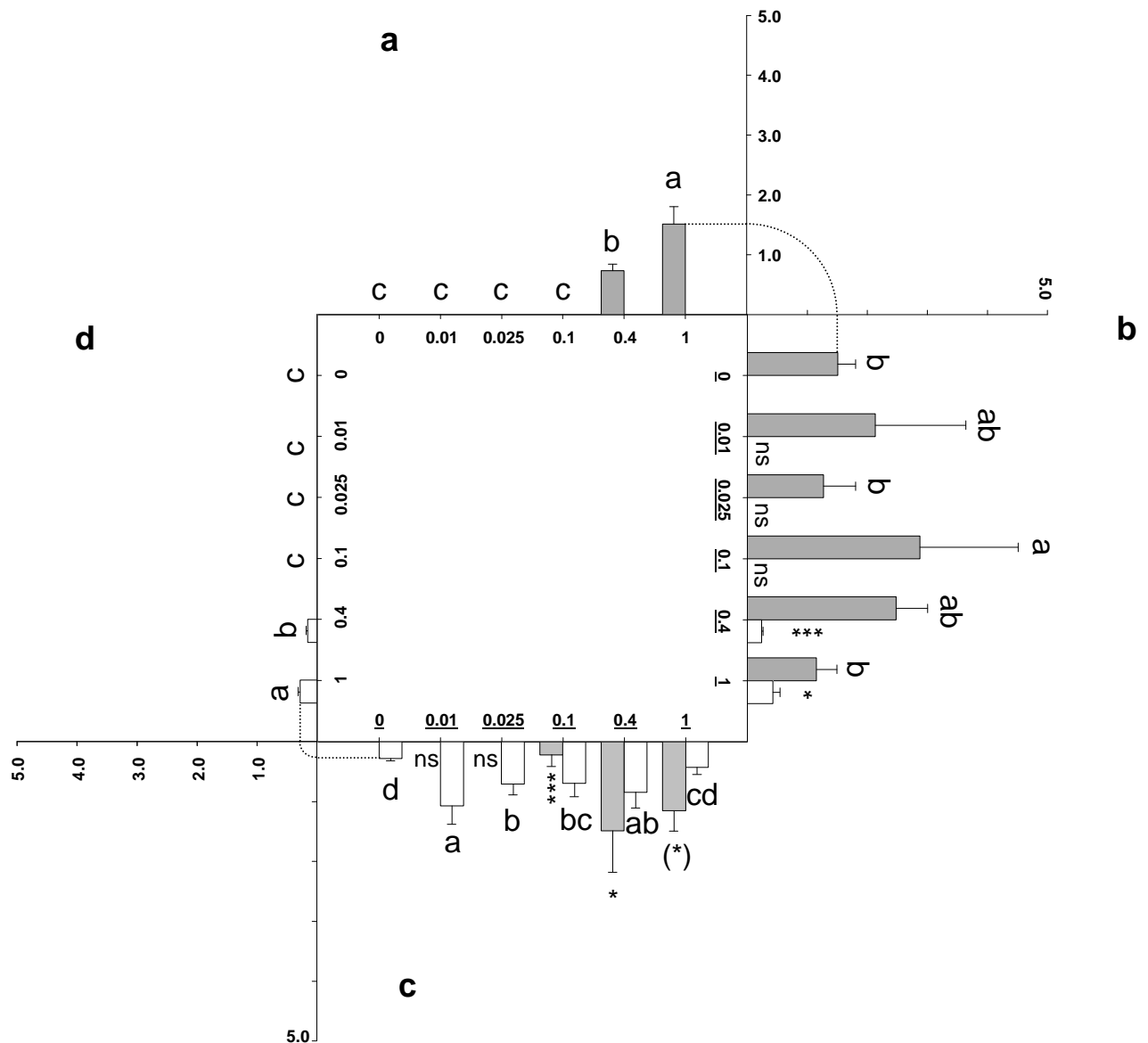
**Figure 4**

Copy numbers of the large ribosomal subunit (LSU) of the two different arbuscular mycorrhizal fungal (AMF) species (millions per mg dry weight of roots) in the roots of medic plants inoculated with different inoculum doses of a single fungal species (a, d) or with fungal mixtures as described in the legend of Figure 1 (b, c). Grey bars represent *Glomus intraradices*, and black bars represent *Glomus claroideum*. Mean values of five replicates + standard deviations are shown. For single inoculation treatment, different letters indicate significant differences between mean values of the different inoculation doses, according to least significant difference-based multiple range test following significant ANOVA ($P < 0.05$). For dual inoculation treatments, different letters indicate significant differences between mean values of the different dual inoculation treatments, where the original inoculum density of the tested fungal species was always the same.

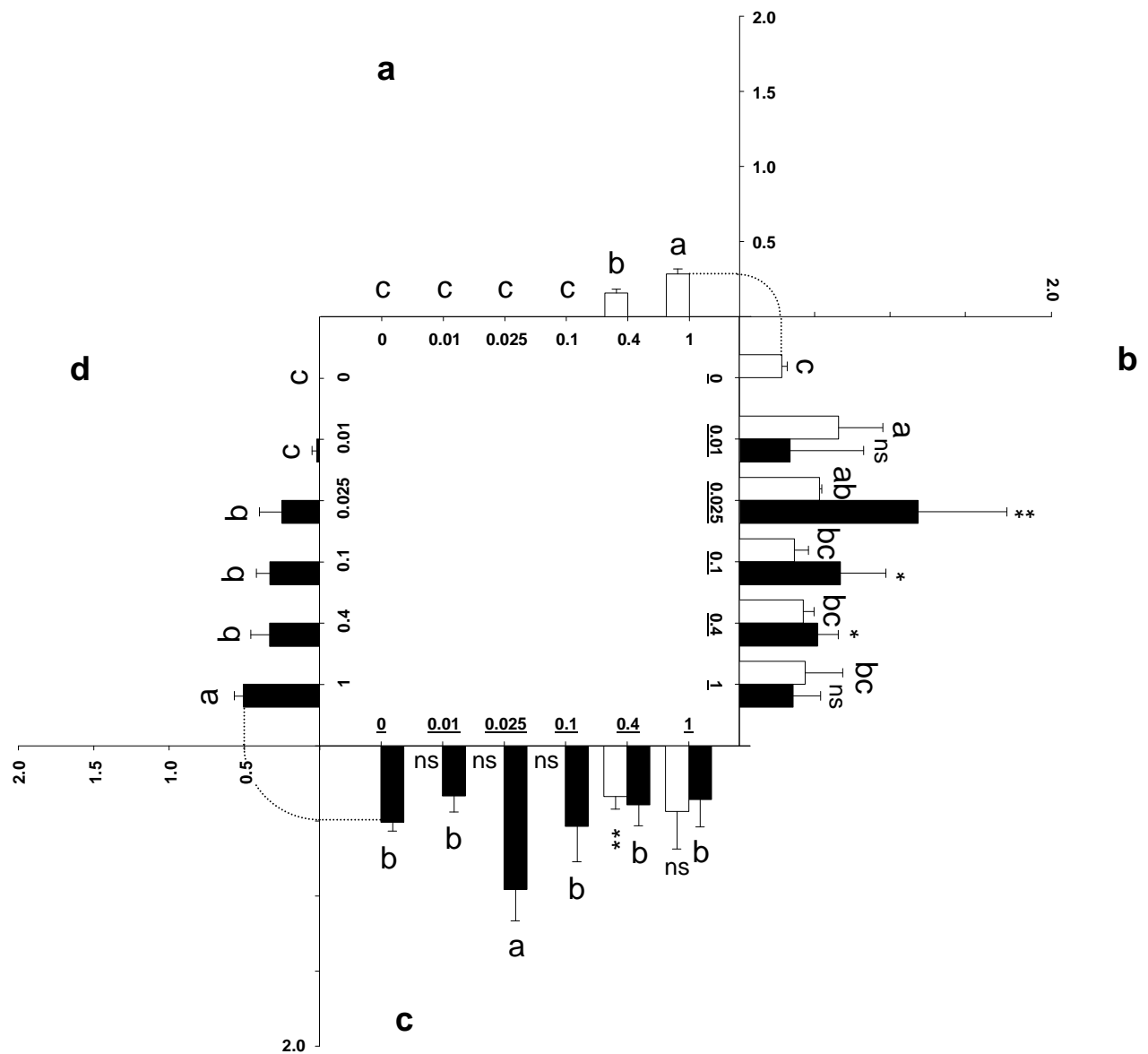
Further, a Student's *t*-test has been performed to test whether the LSU copy numbers were different for the corresponding inoculum densities of the same AMF species inoculated either singly or in a mixture with the other AMF species. Outcome of this comparison is labeled as follows: ns – not significant; (*) $0.05 \leq P < 0.1$; * $0.01 \leq P < 0.05$; ** $0.001 \leq P < 0.01$; *** $P < 0.001$).

Plants co-inoculated with 100% inoculum density of *Glomus intraradices* and increasing inoculum density of *Gigaspora margarita* showed no significant decrease of LSU copy number of *Glomus intraradices* (Fig 5b, grey bars). For some combinations, LSU copy numbers of *G. intraradices* were even significantly higher than for the single inoculation, but with no correlation to the inoculum density or to the abundance in the roots of *Gigaspora margarita* (Fig 5b, white bars). Abundance in roots of *Gigaspora margarita* was significantly increased in the presence of 100% inoculum density of *Glomus intraradices* compared to the respective single inoculation treatments (Fig 5b and 5d, white bars). Plants co-inoculated with 100% inoculum density of *Gigaspora margarita* and increasing inoculum density of *Glomus intraradices* showed a significant increase of LSU copies number of *Gigaspora margarita* in most of the dual inoculation treatments as compared to the single inoculation (Fig 5c, white bars), but these increases did correlate neither with the inoculum density nor to the abundance in the roots of *Glomus intraradices*. The latter eventually reached higher abundances in roots co-inoculated by 100% inoculum density of *Gigaspora* and 40% and 10% inoculum density of *Glomus intraradices* as compared to the respective single inoculations by the *Glomus* (Figs 5a and 5c, grey bars). Notably, no detectable colonization by *Glomus intraradices* was measured when inoculated singly with 10% inoculum density (Fig. 5a), but detectable colonization levels were encountered when in combination with *Gigaspora* (Fig. 5c).

Plants co-inoculated with 100% inoculum density of *Gigaspora margarita* and increasing inoculum densities of *Glomus claroideum* showed a significant increase of LSU copy number of *Gigaspora margarita* if in combination with highly diluted (1% and 2.5%) *G. claroideum* inoculum (Fig 6b, white bars). The abundance in the roots of the latter was sometimes enhanced by the presence of 100% inoculum density of *Gigaspora margarita* as compared to the single inoculations treatments (Fig 6b and 6d, black bars).

**Figure 5**

Copy numbers of the large ribosomal subunit (LSU) of the two different arbuscular mycorrhizal fungal (AMF) species (millions per mg dry weight of roots) in the roots of medic plants inoculated with different inoculum doses of a single fungal species (a, d) or with fungal mixtures as described in the legend of Figure 1 (b, c). Grey bars represent *Glomus intraradices*, and open bars represent *Gigaspora margarita*. Mean values of five replicates + standard deviations are shown. For description of statistical comparisons and symbols, refer to the legend of Figure 4.

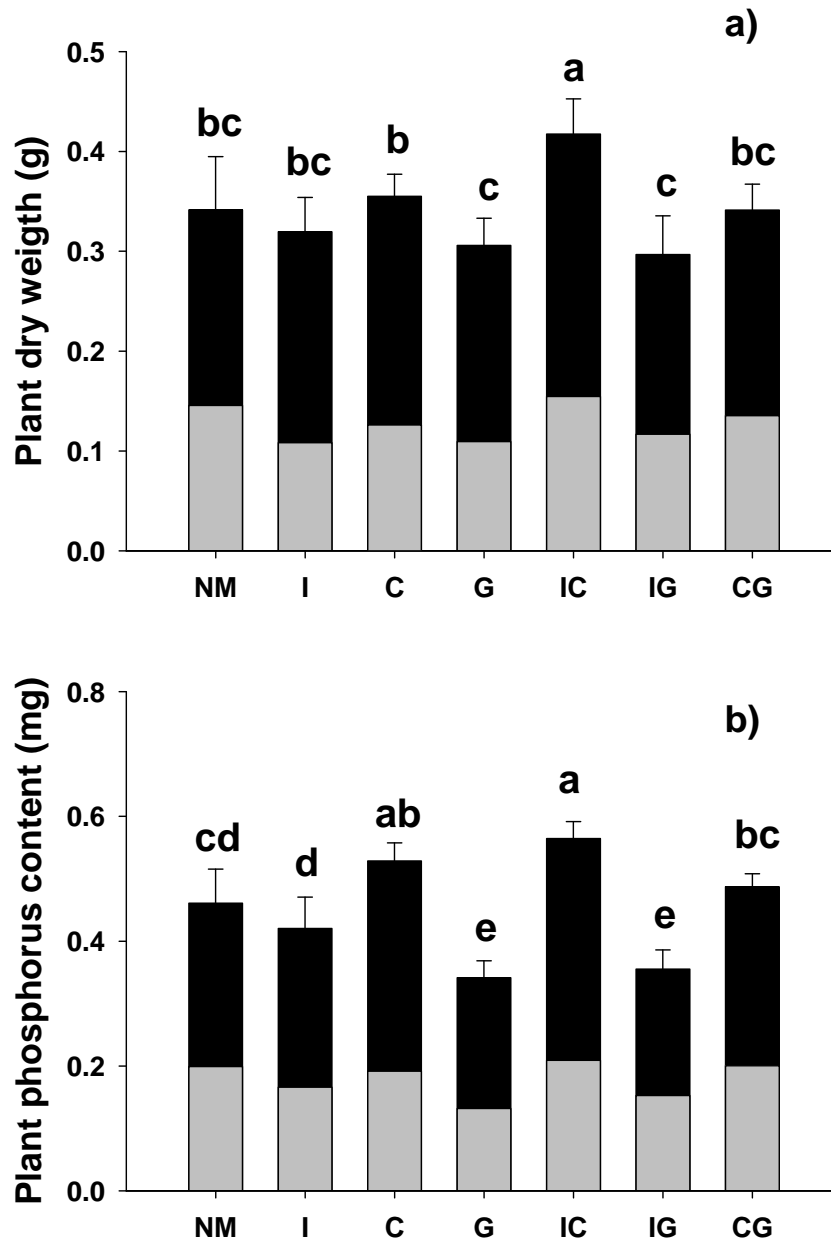
**Figure 6**

Copy numbers of the large ribosomal subunit (LSU) of the two different arbuscular mycorrhizal fungal (AMF) species (millions per mg dry weight of roots) in the roots of medic plants inoculated with different inoculum doses of a single fungal species (a, d) or with fungal mixtures as described in the legend of Figure 1 (b, c). Black bars represent *Glomus claroideum*, and open bars represent *Gigaspora margarita*. Mean values of five replicates + standard deviations are shown. For description of statistical comparisons and symbols, refer to the legend of Figure 4.

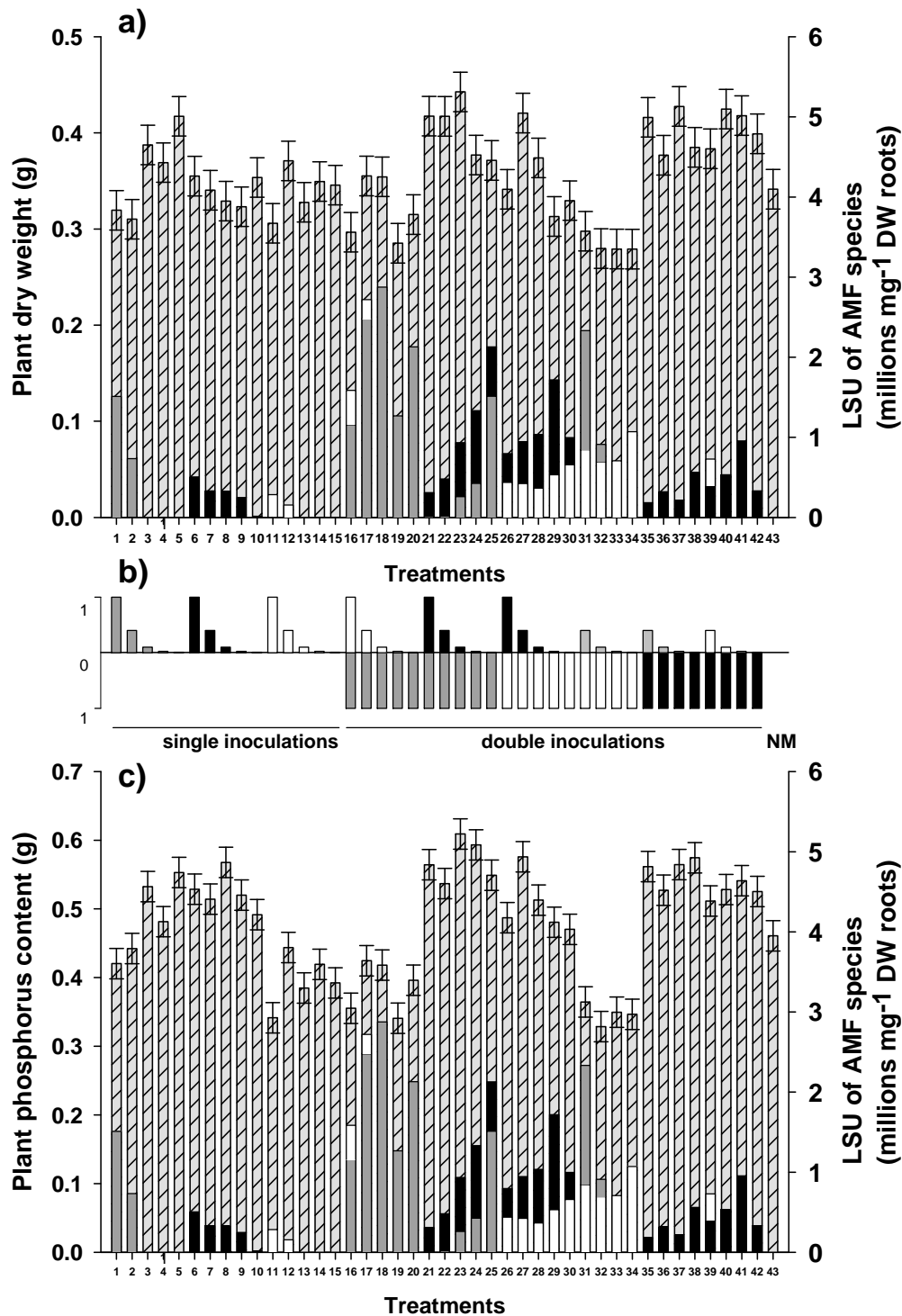
Plants co-inoculated with 100% inoculum density of *Glomus claroideum* and increasing inoculum densities of *Gigaspora margarita* showed no significant difference of LSU copy numbers of *Glomus claroideum* among the treatments (Fig 6c, black bars) with an exception for the plants inoculated with 100% inoculum density of *Glomus claroideum* and 2.5% inoculum density of *Gigaspora margarita*. Surprisingly, exactly in this level of inoculation by the *Gigaspora*, no measurable colonization by the *Gigaspora* was observed either when inoculated alone or in combination with the *Glomus* (Figs. 6a and 6c).

Relationships between AMF community composition and plant growth and P uptake

Compared to non-mycorrhizal treatment, single AMF inoculations with 100% inoculum density did not lead to any benefit in plant growth improvement (Fig 7a). Plants that were co-inoculated with 100% inoculum density of *Glomus intraradices* and *Glomus claroideum* (IC) showed a significantly higher plant biomass compared to NM plants, whereas no difference to the NM plants was observed for the two other dual inoculations (IG and CG). More differences between the different inoculation treatments were observed on the plant P uptake (Fig. 7b). Significant differences were observed between the three different AMF species, with *Glomus claroideum* increasing plant P content and *Gigaspora margarita* decreasing it as compared to the NM plants. *Glomus intraradices* did not affect plant P uptake as compared to the NM treatment. For double inoculations treatments, higher P content was measured in plants inoculated with *Glomus claroideum* and *Glomus intraradices* as compared to the NM plants, whereas the combination of *Glomus intraradices* and *Gigaspora margarita* led to a lower P content as compared to the NM plants. Additional results including all different inoculation treatments further confirmed that the presence of *Glomus claroideum* in the roots improved both the plant biomass and their phosphorus content while the presence of *Gigaspora margarita* in the roots was responsible for growth depression and lower P content as compared to the NM plants (Fig. 8a and 8c). Plants inoculated only with *Glomus intraradices* did not exhibit any growth or phosphorus uptake benefits. However, when *G. intraradices* was co-inoculated with *Glomus claroideum*, trends were observed towards mutual enhancement of the benefits due to *G. claroideum* inoculation, in spite of the low abundance of the *G. intraradices* in the roots.

**Figure 7**

Combined shoot (black) and root (grey) biomass (a) and phosphorus contents (b) of medic plants inoculated with the highest inoculum dose (level "1") of *Glomus intraradices* (I), *Glomus claroideum* (C), or *Gigaspora margarita* (G), or with their mixtures (IC, IG, CG). NM – non-mycorrhizal treatment. Mean values of five replicates + standard deviations are shown. Different letters indicate significant differences between the means according to least significant difference-based multiple range test following significant ANOVA ($P < 0.05$).

**Figure 8**

Dry weight (a) and phosphorus content (c) of medic plants 42 days after sowing. Plant were inoculated (b) by single arbuscular mycorrhizal fungal (AMF) species (treatments 1 to 15), or by two-species mixtures (treatments 16 to 42), or not inoculated (treatment 43) as described in the Material and Methods section and illustrated in Figure 1. Three AMF

species were included in this study: *Glomus intraradices* (grey bars), *Glomus claroideum* (black bars) and *Gigaspora margarita* (open bars). Mean values of five replicates \pm global estimate of the least significant difference (following multiple range test of significant ANOVA ($P < 0.05$)) of the dry weights and plant phosphorus contents are shown (hatched bars). Grey, black and open bars represent absolute values of LSU copy numbers (millions per mg dry weight of roots, right axis) of *Glomus intraradices*, *Glomus claroideum* and *Gigaspora margarita* respectively, measured by real-time PCR in the roots at harvest.

Discussion

Root colonization of single inoculations:

The objective of the adjustment of the inoculum density was to achieve, after 6 weeks, approximately 50% of root length colonized by any of the three different AMF species, when inoculated singly and with the highest inoculum density. Data obtained previously (chapters 1 and 2) allowed determining these densities and have shown that both inocula of *Glomus intraradices* and *Glomus claroideum* had to be highly diluted to reach this objective. The serial dilutions of these level “1” inocula had for consequences that for *Glomus intraradices* values below 1 spore per container were reached. Different studies, including the results obtained within this PhD (see previous chapters), that have analyzed the root colonization of plants by *Glomus intraradices*, indicated that reducing *G. intraradices* infectivity to reach 50% RLC is an extreme case and cannot be regarded as normal situation (Jansa *et al.*, 2005; Jansa *et al.*, 2008). Therefore and regardless the presence of other propagules in the inoculum like mycelium fragments and pieces of colonized leek roots, the attempt to further reduce the degree of root length colonization by *Glomus intraradices* employing a serial dilutions may have resulted in a significant decline in infectivity simply by diluting out the last infective propagules. This would explain why colonization of roots was only observed for the less diluted inocula of *Glomus intraradices* (level 1 and 0.4 see Fig. 2b) and why the lag phase (period during which colonization stays low before exponential increase) of the “full” inoculum (level 1) was so long (Fig. 2a). Indeed, other experiments using the same isolate of *Glomus intraradices* have shown that the RLC levels achieved in as short time as 4 weeks are usually close to 100% regardless of variation in inoculum infectivity (first chapter and (Jansa *et al.*, 2008))

The roots obtained from the single inoculation treatments, harvested at different times after sowing, were submitted to DNA extraction in order to quantify LSU copies of the AMF species in the roots (Fig. 2c). The correlations of LSU concentrations in roots with the RLC measured by microscopy (Fig. 3) confirmed the results obtained in the first chapter with the exception of *Glomus claroideum*, for which a negative correlation between these two methods of colonization assessment was previously shown. Nevertheless, as it was discussed in the first chapter, assessment of root colonization by real-time PCR provides confident results when comparing plants of the same stage (as it is the case in this experiment). Additionally, the real-time PCR most probably better reflects the metabolically active mycelium as well as the colonization intensity (number of hyphae per root intersection) than the traditional assessment by microscopy. Based on that, we were confident using the LSU copy numbers for assessing the outcomes of interspecific interactions between the different AMF species.

Because the numbers of LSU copy per unit of root dry weight for *Glomus intraradices* is several time higher than for the other two AMF species, it was decided to compare these LSU values for one species at a time for the different double-inoculation treatments. This variation in absolute values of LSU copies was already observed in the studies of Jansa *et al.* (2008) and Alkan *et al.* (2006) and was discussed in the first chapter. It probably reflects differences in genome organization by the different AMF species.

Interspecific competition

When investigating interactions between different AMF species, the quantity of inoculum of each fungus has to be considered so that the relative infectivity of the fungi is known; otherwise a species may be outcompeted during colonization solely because it had relatively low numbers of infective propagules. In this experiment, one AMF species was challenged by five levels of inoculum of its competitor in order to cover a range in the infectivity of this latter (additive design (Weigelt & Jolliffe, 2003)). Similar approach and design were employed in the works of Pearson *et al.* (1993) and of Wilson and Trinick (1983), but these previous studies were not using quantitative methods for assessment of colonization by the different AMF species such as the real-time PCR. Some other studies have usually used a different design (replacement series) where the total number of

infective propagules was maintained in the mixed inoculum but the ratio of each interacting species was varying (Wilson, 1984b).

Results obtained in the present additive design seem to show two kinds of interactions. The first one concerns the interaction between *Glomus claroideum* and *Glomus intraradices* and the second the interactions between *Gigaspora margarita* and any of the two *Glomus* species.

Interaction between *Glomus claroideum* and *Glomus intraradices* (Fig. 4):

Plants co-inoculated with full inocula of these two species show after 42 days an almost complete repression of the development of *Glomus intraradices* in the roots. This phenomenon nearing a total competitive exclusion of one AMF species by another has already been observed (Hepper *et al.*, 1988) for two species of *Glomus*. Data given by the single inoculations (Fig. 2) indicate that roots were probably colonized first by *Glomus claroideum*. This prior root occupancy is explained by the used inoculum properties of *Glomus claroideum* whose density of infective propagules was higher. Nevertheless, medic roots are able to accommodate more than 50% root length colonization and, therefore, roots could have been potentially colonized by *Glomus intraradices* unless competition occurred between the two *Glomus* species. This observed competition might take place already in the rhizosphere where germination of *G. intraradices* propagules could have been inhibited either directly by its competitor in the soil via hyphal exudates or by the colonized roots whose exudates have been shown to be involved in the pre-mycorrhizal infection stages (David-Schwartz *et al.*, 2003) and to be altered in the study of Pinor *et al.* (1999) to prevent further colonization. Another possibility is that colonized roots by *Glomus claroideum* are less susceptible to penetration by germinating hyphae of *Glomus intraradices*. This phenomenon could be mediated by mechanisms of plant defense that would make the root cells immune to further mycorrhizal colonization. The participation of the plant in these mechanisms of competition between AMF has been shown to act locally and also in distant root zones through systemic plant signaling. Split-root systems such as developed in the study of Vierheilig (2004a) have shown that pre-inoculation of roots on one half of the system with an AMF isolate (*Glomus mosseae*) prevents further colonization of roots on the second half of the split-root system.

Jasmonic acid is thought to be involved in this mechanism (Herrera-Medina *et al.*, 2008). Recently, it has been also shown that a similar pre-inoculation of medic roots had conducted to enhancement of expression of genes involved in plant defense mechanisms in roots of the second half of the split-root system (Liu *et al.*, 2007).

Interestingly, these mechanisms mediated by the plant seem to be dependent of the identity of the interacting fungi. Indeed, prior occupancy of roots by *Glomus claroideum* does not affect the development of *Gigaspora margarita*, which on the contrary to *Glomus intraradices* was facilitated (Fig 6).

The possibility of specific signaling at the plant level is also corroborated by that fact that the development of *Glomus intraradices* (inoculated with level “1”) is nevertheless still repressed even if no prior occupancy of the roots by *Glomus claroideum* can be substantiated, e.g. in the case with inoculum diluted 40 times (level “0.025”) (Fig 4b).

In total, the outcome of the interactions between these two *Glomus* species shows that *Glomus claroideum* is much more aggressive than *Glomus intraradices*. This would support the theory of Wilson that claims that aggressiveness appeared to be inversely proportional to infectivity (1983 and 1984b). The study of Jansa *et al.* (2008) has used the same isolates of *Glomus* species in an experimental design where similar amounts of fresh and non diluted inocula of *Glomus intraradices* and *Glomus claroideum* were mixed and inoculated to leek and medic plants. The LSU copy numbers contained per root mass was estimated by real-time PCR after 4 and 8 weeks of culture and results showed almost an equivalent colonization of roots by the two *Glomus* species after 4 weeks but then a dominancy of *Glomus intraradices* in the roots after 8 weeks. These results contradict our findings but can probably be explained by a faster colonization of the roots by *Glomus intraradices*, due to a higher density of infective propagules of its inoculum and to other factors shown to influence colonization like, among others, growth conditions and phosphorus availability of the substrate (Daft & Hogarth, 1983; Alkan *et al.*, 2006).

Therefore we can conclude that interactions between the two *Glomus* species would normally give on one side the advantage to the faster colonizer; property influenced by the density, the distribution and the state of activation of their infective propagules; and on the other side when these factors are manipulated to invert the order of root occupancy

(like in the present experiment) the disadvantage to *Glomus* species with the highest infectivity.

Interactions between *Gigaspora margarita* and *Glomus* species (Figs. 5 and 6):

Plants co-inoculated with full inocula (level “1”) of both *Gigaspora margarita* and *Glomus intraradices* or *Glomus claroideum* show after 42 days the same pattern of interactions. Development of the two *Glomus* species is not affected by inoculation of *Gigaspora margarita* whose root occupancy is facilitated. A possibility explaining this situation might be that *Gigaspora* and the *Glomus* species would preferentially colonize different zones of the root system and, therefore, would not compete for root occupancy. Carbon cost of the symbiosis between medic plants and *Gigaspora* has been shown to be higher than with the other two *Glomus* species (see previous chapter and work of Lendenmann *et al.* (in preparation)). In the same time, colonization of roots by *Gigaspora* species (Lerat *et al.*, 2003) has been shown to enhance the “sink” demand for carbon, which would have created in this experiment a gradient of concentration between the shoot and the roots. This gradient could have resulted in a preferential spread of *Gigaspora margarita* towards the root zones close to the shoot where the concentration of photosynthates is higher and would explain the differential occupancy of the roots by *Gigaspora* and the two *Glomus* species. Yet, the theory of occupation of different root zones by different AMF species remains to be confirmed experimentally.

Together, the absence of competition and evidence for facilitation and the hypothetical differential root occupancy of *Gigaspora margarita* and *Glomus* species would support the idea that plants promote the occupancy of their roots by phylogenetically unrelated and/or functionally different AMF species. This was already suggested by Maherali and Klironomos (2007), who proposed that such AMF communities in the roots would be more beneficial for the plant because of functional complementarity such as uptake of nutrients in different spatial zones or in different times (which would explain why AMF with different life history strategies would become promoted when co-occurring). The fact that facilitation events have occurred even when the competitor species was not detected in the roots would indicate that signaling not only takes place in the roots but also outside, in the rhizosphere.

The hypothesis of differential root occupancy could easily be tested by using the present molecular assay on different root fragments of the root system and the method developed in the study of Seddas *et al.* (2008b) based on *in-situ* PCR could even allow determining more precisely the extent of co-occurrence of these AM species. Absence of differential root occupancy for *Gigaspora* and *Glomus* would involve the AM fungi as well as actors of facilitation events through possible production of stimulating compounds.

Consequences of mycorrhizal interactions for plant growth and P uptake

Results obtained for the plants responses in this experiment should be interpreted carefully because the size of the containers has probably limited not only the root growth but also the growth of the mycorrhizal mycelium, which is a key feature of the symbiotic efficiency as it was largely documented in the literature (Avio *et al.*, 2006; van der Heijden & Scheublin, 2007) and in the previous chapter. Nevertheless, it is interesting to note that in spite of this limitation, significant differences observed between the inoculation treatments (Figs. 7 and 8).

When inoculated singly and with the highest doses (level “1”, Fig. 7), plant biomass was not significantly different from the non-mycorrhizal treatment while the plant P content was improved by *Glomus claroideum* and reduced by *Gigaspora margarita* inoculations. The low P content of plants colonized by *Gigaspora margarita* confirms the tendency observed in the previous chapter, where it has been shown that P might be stored in the mycorrhizal hyphae before being delivered to the plants. Surprisingly, *Glomus intraradices* inoculated singly did not improve the plant P content or the plant biomass while this AM isolate had been shown to provide such benefits even when colonizing the roots at 50% (see first chapter). Results obtained by Jansa *et al.* (2005 and 2008) and the results contained in the previous chapter have demonstrated that the efficiency of this isolate was mainly in the ability of its mycelium to forage for P far from the roots. The limited size of the containers might then explain why *Glomus intraradices* could not demonstrate its efficiency – simply because there was nowhere to grow. Nevertheless, when this isolate was co-inoculated with *Glomus claroideum*, the effects on plant growth and P content were usually superior to the effects exerted by the respective single AM species (Fig. 8a and 8c). This finding is supporting the theory of functional complementarity with respect to P acquisition between AMF species colonizing roots, as

suggested by Koide (2000) and demonstrated by Jansa *et al.* (2008) for the same combination of AMF isolates.

Interestingly, this complementary effect is even observed in treatments where very few LSU copies of *Glomus intraradices* were measured, because the inoculum was heavily diluted and also because the other *Glomus* had obviously a competitive advantage.

Together, the results presented above demonstrate a discrepancy between the finding that 1) in co-inoculation treatments, medic plants would promote root occupancy of *Gigaspora margarita* and any of the two *Glomus* species and that 2) inoculations of species that are competing (*Glomus claroideum* and *Glomus intraradices*) would provide the best benefits in terms of plant biomass improvement and phosphorus acquisition.

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General discussion, conclusion and outlooks

The general objective of this thesis was to create a proper framework together with a model system allowing a better understanding of factors governing the establishment and functioning of mycorrhizal communities. To achieve this goal, an important effort was made to develop specific detection tools enabling specific quantification of AM fungi in colonized roots and in the soil. The use of a model system (plant and substrate), reproducible and coupled with controlled conditions have enabled to dissect with more precision the morphological and physiological traits of AMF playing a role in their establishment and functioning, when they are present as a community.

The functions of a mycorrhizal community are directly influenced by the identity of the AMF species that are first competing for their establishment in the roots, from where they will start producing their extraradical mycelium whose features (morphological and physiological) will determine the extent of plant responses in terms of P acquisition and plant biomass production. In the frame of this thesis, the model system and the conventional and newly developed methods have allowed characterizing the determinants of the mycorrhizal symbiosis that will govern the establishment and the functioning of a mycorrhizal community (Fig. 1).

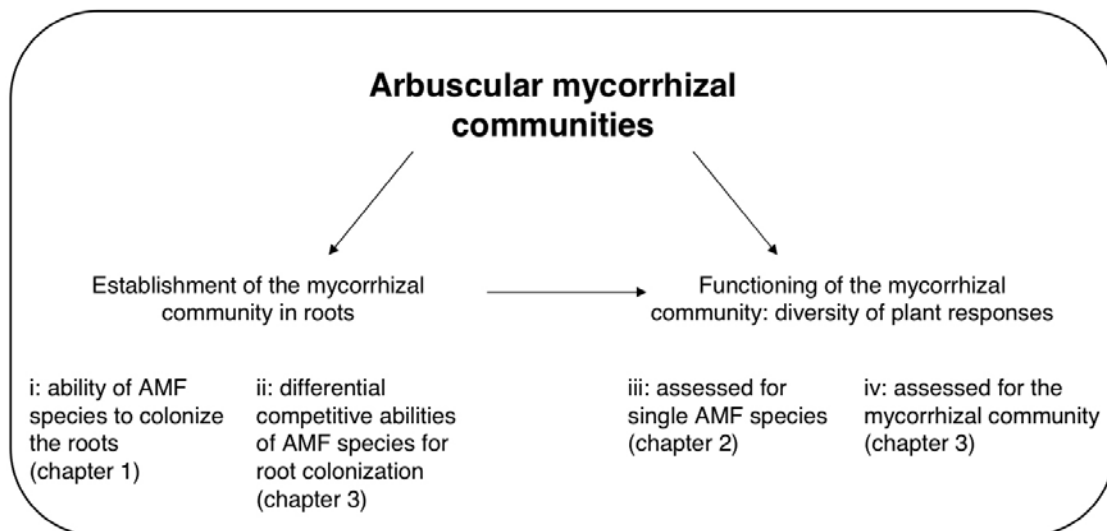


Figure 1
Scheme representing the main topics addressed in the frame of this PhD project.

Establishment of a mycorrhizal community

If we consider a plant co-inoculated with different AMF species, the results presented in chapters 1 and 3 show that the establishment of a mycorrhizal community in roots is mainly dependent on (i) the ability of the different AMF species to colonize the root and on (ii) their ability to compete for root colonization.

- i. The dynamics of root colonization of five different AMF fungi was analyzed in chapter 1. We know from the literature that many factors are influencing the ability of an AM species to colonize roots. These are mainly the environmental factors, the plant species and the properties of the mycorrhizal inoculum (Klironomos & Hart, 2002; Drew *et al.*, 2003; Smith & Read, 2008) (Fig. 2). Following standardization of these factors, the results presented in chapter 1 showed diversity in the root colonization patterns of the studied AM fungi. This diversity was expressed in differential rate, extent and intensity of colonization, and in differential dependence of the colonization on the density of infective propagules of their inoculum. *Glomus intraradices* and *Glomus claroideum* were shown to be fast root colonizers (refer to the rate of colonization) but with a different maximum extent of the colonization. Their ability to colonize the roots was not much influenced by the density of their infective propagules in this study. This lack of dependency was also observed for *Glomus mosseae*, which was a slower colonizer, but with an intermediate extent of root colonization. On the contrary, ability of root colonization of *Gigaspora margarita* and *Scutellospora pellucida* was influenced by their inoculum densities (chapter 1). These different abilities of AMF to colonize the roots were assessed by the traditional staining-microscopy method (Brundrett *et al.*, 1984; McGonigle *et al.*, 1990) and by measurement of LSU copy numbers using the new molecular tools. The results showed the occurrence of discrepancies between the two methodical approaches indicating that LSU copy numbers were probably better reflecting the symbiotic activity than the traditional method. The responses of plants in terms of P acquisition and biomass production were shown not to be completely governed by the extent of root colonization. Nevertheless, the time-course assessment of the shoot P uptake benefits (it means in comparison to the non-mycorrhizal plants) supported the idea that the molecular assay was more appropriate to assess the active fungal biomass in roots. The differential ability of an AM fungi to colonize the roots is

also thought (Hart & Reader, 2005) to be influenced by its mode of infection spread as it is discussed in chapter 1. Further, colonization spread could also be limited by occurrence of phenomenon of intraspecific competition (Wilson & Tommerup, 1992) that would explain why the extent of root colonization is limited in some AM fungi, like it seems to be the case for *Glomus claroideum*. Finally, the results presented in chapter 1 have allowed to manipulate the conditions (inoculum density, time of harvest) in the other parts of the research presented here in order to enable the comparison of other mycorrhizal traits known to play a role in the diversity of the plant responses. Following these standardization measures it was possible to obtain roots colonized to a similar extent in experiments whose results are presented in chapter 2 and 3.

- ii. The establishment in roots of a mycorrhizal community is also influenced by the differential ability of the co-inoculated AM fungi to compete for root colonization (Fig. 2). The literature on that topic indicates that the environmental factors and the host plant species (Alkan *et al.*, 2006; Jansa *et al.*, 2008) influence the outcome of competition occurring between interacting fungi as they do for their root colonization ability. The results presented in chapter 3 strongly highlighted the competitive abilities of AM fungi as being governed by the identity of the interacting fungi. It is assumed that interacting fungi will compete for space (Wilson & Tommerup, 1992) and for resources (Pearson *et al.*, 1994) (Fig. 2), but the differential combinations of AM fungi co-inoculated to medic plants have also provided several pieces of evidence for facilitation processes. Interestingly, *Glomus intraradices* and *Glomus claroideum* were competing (with *Glomus claroideum* being the best competitor) while the development in roots of *Gigaspora* was enhanced in the presence of any of these two *Glomus*. As it was discussed in chapter 3, the results of these interactions are probably mediated by the plant and/or by the interacting AM fungi directly (Fig. 2). The fact that one AM fungus is occupying the root as first due to a higher rate of root colonization (like *Glomus claroideum*) might explain on one hand its better competitive ability when interacting with the other AM fungus (like *Glomus intraradices*) that either targets the same root zone (competition for space (Hepper *et al.*, 1988)) or is phylogenetically too close (repression possibly mediated by the plants that would promote occupancy of its roots by functionally different AM fungi (Maherali & Klironomos, 2007)).

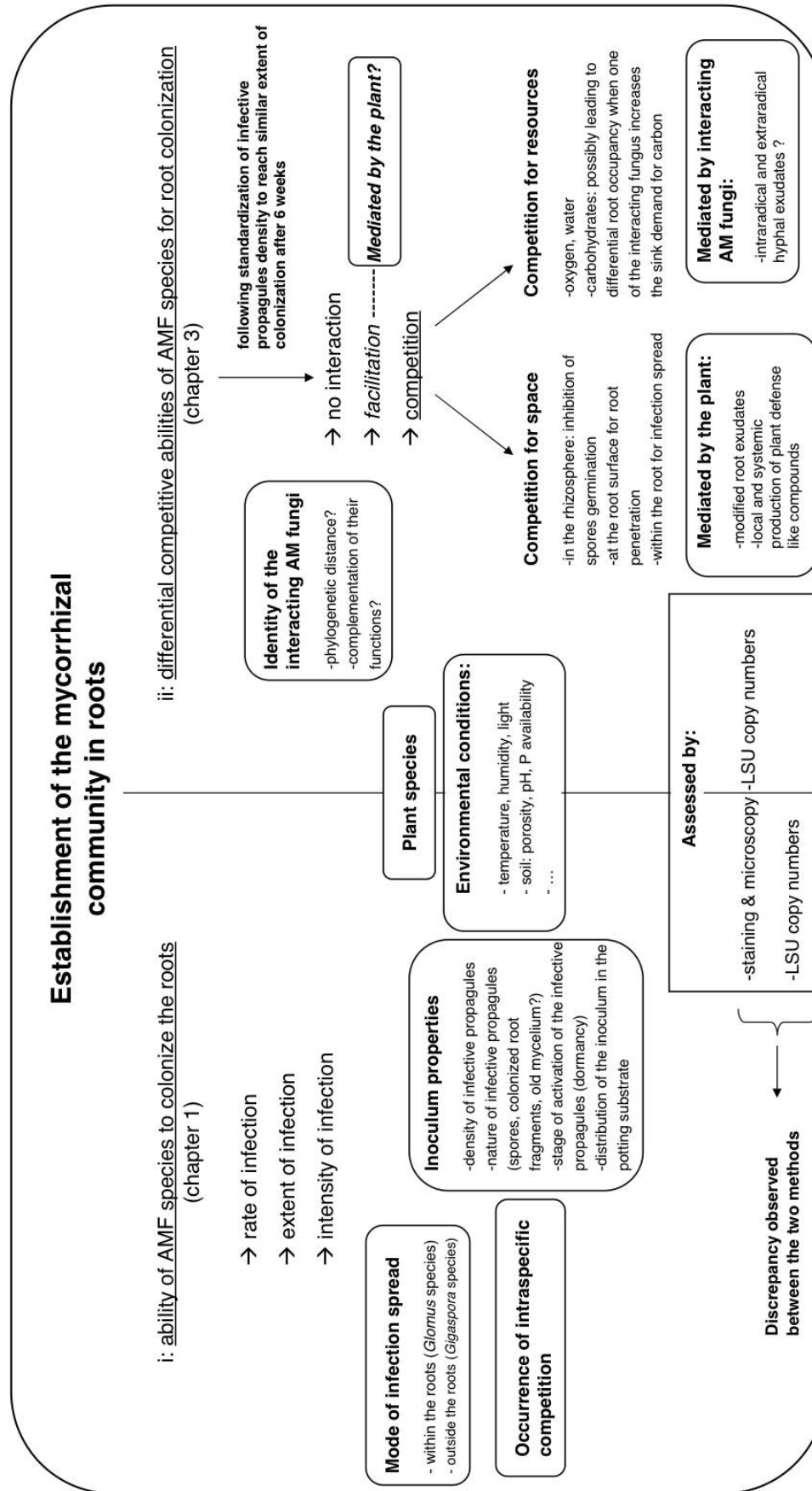


Figure 2: Scheme representing the important factors involved in the establishment in roots of a mycorrhizal community (see text for details)

On the other hand this prior root occupancy could promote the development of the other AM fungus (like *Gigaspora margarita*) that is either colonizing different zones of the root system (no competition for space due to differential root occupancy) or is providing other kinds of benefits because of being functionally different (facilitation also possibly mediated by the plants).

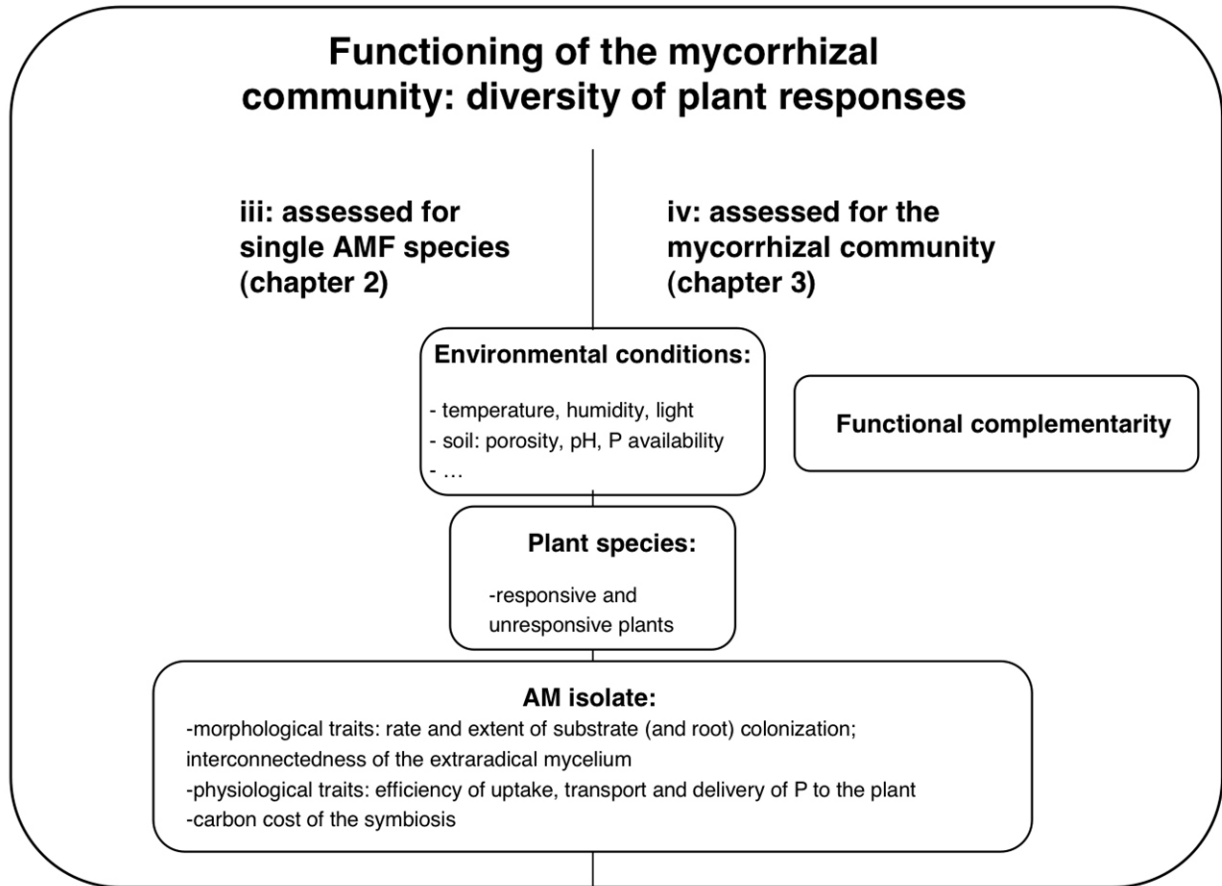
Functioning of a mycorrhizal community

Characterizing and understanding the functional diversity of mycorrhizal communities should start with (iii) the assessment of the varying responses of plants when inoculated with single AM species and in regards to their morphological and physiological properties. Following this assessment, the impact for the plant of multiple colonizations can be analyzed (iv) and further understood when knowing the relative abundance in roots of the different co-inoculated AM fungi (see above). The ideas and concepts are summarized in Figure 3.

- iii. The results presented in chapter 2 were highlighting the mycorrhizal traits and features explaining the diversity of plant responses following establishment of the symbiosis. As discussed in chapter 2, these mycorrhizal traits were mainly concerning 1) the morphological properties of the extraradical mycelium that demonstrated different abilities to colonize the substrate (at different rate and extent) and 2) the physiological properties of the AM fungi whose efficiency to take up, transport and deliver phosphorus to the plant was showing a significant level of diversity. In addition, 3) the differential requirement of carbohydrates of colonizing AM fungi was also shown in this chapter and in the work of Lendenmann et al. (in preparation) to explain the diversity of plant responses. Together, these experiments showed that the different AM fungi have a different strategy to acquire P and to improve the plant biomass. More into details, these assessments, that were successfully carried out with the use of labeled phosphorus (^{32}P and ^{33}P) and carbon (^{13}C), have demonstrated that *Gigaspora* was gathering P only from limited soil volume, where it was establishing dense mycelium networks, and that the P taken up from the soil was to some extent immobilized in the fungal mycelium before delivered to the plants. Also, it was shown that *Gigaspora* drained large amounts of carbon from the plants and that its colonization resulted, as a

consequence, in negligible overall benefits to plant growth as compared to non-mycorrhizal plants. The extent of soil exploration by *Glomus intraradices* and *Glomus claroideum* was higher than for *Gigaspora* and the first *Glomus* species was shown to provide more P to the plants. The lower carbon demand of *Glomus claroideum* (as compared to *Glomus intraradices*) was explaining why the levels of growth promotion of these two *Glomus* species were eventually similar.

- iv. Results presented in chapter 3 indicated that plants had varying responses to multiple colonizations of their roots. In general, the P content and the biomass production of plants colonized by *Glomus claroideum* were enhanced when *Glomus intraradices* was co-inoculated. As discussed in chapter 3, these results are supporting the theory of functional complementarity with respect to P acquisition between AMF species colonizing roots, as suggested by Koide (2000) and demonstrated by Jansa *et al.* (2008) for the same combination of AMF isolates as used in this PhD project. On the contrary, plants colonized by *Gigaspora margarita* (single inoculated or co-inoculated with any of the two *Glomus* species) had a tendency to have their biomass and P content reduced compared to plants not harboring this species in their roots. This finding can be understood in the light of elements brought by results presented in chapter 2, validating the approach according to which functioning of mycorrhizal communities should be analyzed after having first characterized properly and separately the functions of AMF species composing the mycorrhizal community.

**Figure 3**

Scheme representing the important factors playing a role in the functioning of a mycorrhizal community (see text for details).

Conclusion and outlooks:

In the frame of this PhD project, we have brought new elements to the understanding of the establishment and functioning of mycorrhizal communities. To achieve this, we had to establish a reproducible model system, work under controlled conditions and develop a new set of primers and probes allowing successful quantification of different AMF species co-colonizing the same root system. The results obtained here will open new horizons and provide new perspectives for the understanding of mycorrhizal ecology. The tools are already being used in several other projects and important impacts are anticipated. In chapter 3, these tools have been used to show processes of competition and facilitation between AMF species attempting to colonize the same root system. It would be interesting to evaluate the kind of interactions occurring in the substrate once

the established AMF species produce their extraradical mycelium to gather phosphorus (and other soil-borne resources). A reliable protocol has yet to be established to extract DNA (and RNA) from the soil so that the novel molecular tools could also be used to characterize whether growing hyphae of different AMF species are interacting as they do in the roots. In addition to this characterization, the tools could also be employed to refine the knowledge on the interactions between the AMF species occurring in the roots and more precisely to evaluate whether competing and facilitating fungi are directly (physically) interacting or not. This kind of characterization could be achieved with a direct fluorescent *in situ* RT-PCR (Seddas *et al.*, 2008) using our primers labeled with different fluorescent dyes. This would provide more elements elucidating the participation of the plant in the mediation of the interaction outcomes. Concerning the functioning of mycorrhizal communities, the knowledge gathered during this PhD should allow establishing other mycorrhizal communities in roots, concerning more species and greater levels of diversity. Appropriate quantification of the contribution of species identities and functional diversities within the AMF communities with respect to P uptake and biomass production of the mycorrhized plants would allow modeling this uneasy ecosystem and to eventually allow prediction of the functions of mycorrhizal communities without the need to carry out these tedious experiments.

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