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# Traits related to differences in function among three arbuscular mycorrhizal fungi

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**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. It was grown in a compartmented system with root and root-free zones separated by a fine mesh. Dual radioisotope labeling ( $^{32}\text{P}$  and  $^{33}\text{P}$ ) was employed in the root-free zone as follows:  $^{33}\text{P}$  labeling determined hyphal P uptake from different distances from roots over the entire growth period,

whereas  $^{32}\text{P}$  labeling investigated hyphal P uptake close to the roots over the 48 hours immediately prior to harvest. *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 substrate and transported significant amounts of P towards the roots, but provided the same growth benefit as compared to *Glomus claroideum*, whose

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mycelium was 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 low P benefits to the plants and formed dense mycelium networks close to the roots where P was probably transiently immobilized. Numerical modeling identified possible mechanisms underlying the observed differences in patterns of mycelium growth. High external hyphal production at the root-fungus interface together with rapid hyphal turnover were pointed out as important factors governing hyphal network development by *Gigaspora*, whereas nonlinearity in apical branching and hyphal anastomoses were key features for *G. intraradices* and *G. claroideum*, respectively.

**Keywords** Arbuscular mycorrhiza · Extraradical mycelium · Functional diversity · Hyphal growth model · *Medicago truncatula* · Phosphorus

#### Abbreviations

AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
DW	Dry weight
ERM	Extraradical mycelium
HLD	Hyphal length density
NM	Non-mycorrhizal
P	Phosphorus
<i>p</i>	Probability level
PUE	Phosphorus Use Efficiency
RMSE	Root mean squared error

#### 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 tolerance to environmental stresses and often result in a better plant growth and nutrition, particularly under nutrient deficient conditions (Smith and Read 1997). Following the root colonization, external mycelium is produced in the surrounding soil, where it can take up the inorganic phosphate from the soil solution. Phosphorus (P) is then translocated to the host root and delivered to the plants at the root-arbuscule

interface (Harrison 1999; Karandashov and Bucher 2005). These complex processes imply the action of different transporters and enzymes (of both plant and fungal origin), some of which are already known at the gene level (Javot et al. 2007). Upon establishment of AMF colonization, plants can acquire 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, 2004). This was also supported by modeling studies of Schnepf and Roose (2006) and Schnepf et al. (2008a, b).

In exchange to the P uptake mediated by the fungus, AMF receive from the plant reduced carbon compounds derived from the photosynthesis. It has been estimated that the symbiosis with AMF can cost the plant up to 20% of the plants net photosynthesis production (Jakobsen and 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 terms 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 from the soil solution, transport and deliver it to the roots, along with the carbon requirement from the plant host (van der Heijden and Scheublin 2007). There is a consensus (Avio et al. 2006; van der Heijden and Scheublin 2007) that the differential increases in P supply to host plants are mainly attributed to morphological and physiological properties of the mycorrhizal extraradical mycelium (ERM). The morphological properties refer here to the ability of the ERM to be extensive and interconnected through anastomoses. The physiological properties refer to the viability/overall metabolic activity of the hyphae and, more specifically, to 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 and Scheublin 2007).

To characterize the levels of functional diversity between AMF at a single field site, previous studies focused solely on *Glomus* species (Jansa et al. 2005, 2008) isolated from the Tänikon field site in Switzerland (Jansa et al. 2002). This is now to be amended by including another AMF isolate from the same field site 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 of the mycorrhizal plants. Here we employed a compartmented system (somehow similar to the experimental setup of Jakobsen et al. (1992a, b)) consisting of a plant container and a root-free zone, and we used  $^{32}\text{P}$  and  $^{33}\text{P}$  radioisotope tracing so as to estimate the magnitude of P fluxes in time and space from soil to the plants via the AMF hyphae. Furthermore, we aimed at deciphering the possible mechanisms behind the different patterns of substrate colonization by the different AMF species. This was approached by numerical modeling of the mycelium growth data using the growth model for AMF mycelium developed by Schnepf et al. (2008a).

## Materials and methods

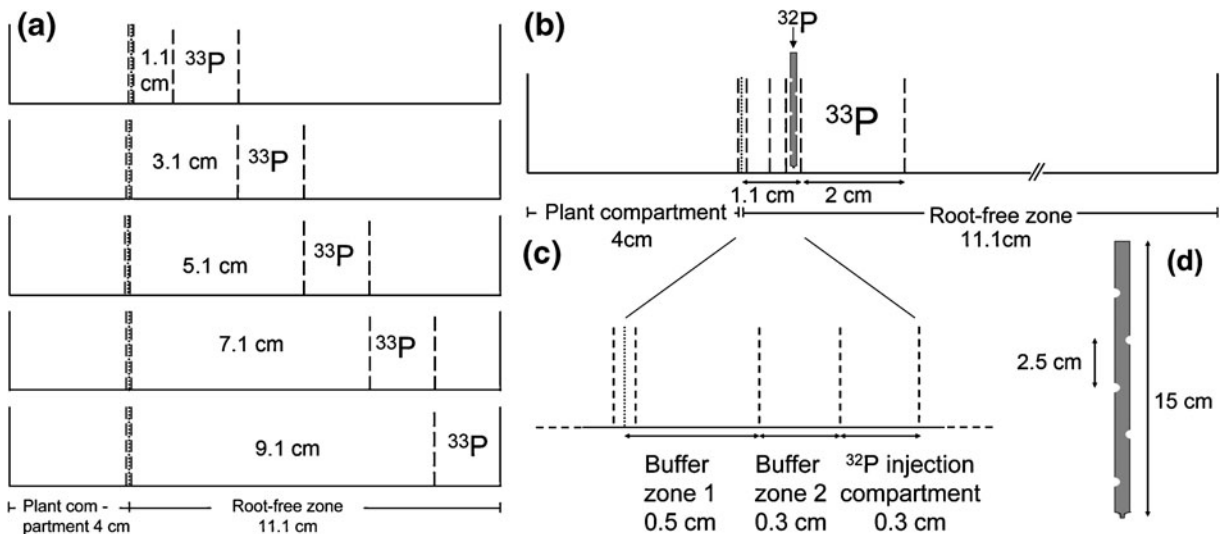
### Experimental setup

The experiment was carried out in compartmented cultivation systems (cuvettes) having plant ( $15 \times 15 \times 4$  cm) and root-free ( $15 \times 15 \times 11.1$  cm) compartments. Both  $^{33}\text{P}$  and  $^{32}\text{P}$  isotopes were applied in the root-free zone (Fig. 1). All zones of the cuvettes were filled with a substrate consisting of a sterilized field soil, sterilized

coarse quartz sand (particle diameter 0.7–1.2 mm) and sterilized fine quartz sand (particle diameter 0.08–0.2 mm), mixed in the ratio of 1:3:1 (v:v:v). The soil was collected in Tänikon, Switzerland (sand 49%, silt 32%, clay 16%), air-dried, passed through 5 mm sieve and  $\gamma$ -irradiated at Studer Hard AG, Däniken, Switzerland, applying a dose of 25–75 kGy with a  $^{60}\text{Co}$  source. The available P content of the substrate was  $21.9 \pm 0.43$  mg  $\text{kg}^{-1}$  (ammonium acetate-EDTA extraction, 1:10 w:v, 16 h), and the readily available P pool ( $E_{1\text{min}}$ ) was  $1.73 \pm 0.06$  mg  $\text{kg}^{-1}$  as assessed by the isotope exchange kinetics approach (Frossard and Sinaj 1997). Substrate C and N contents were  $2.2 \pm 0.1$  and  $0.24 \pm 0.01$  g  $\text{kg}^{-1}$ , respectively. Plant roots were confined into the plant compartment by a 30- $\mu\text{m}$  mesh (Sefar AG, Heiden, Switzerland; Fig. 1b). Root-free zone consisted of four compartments, separated from each other by 500- $\mu\text{m}$  meshes (Sefar). The two buffer zones and  $^{32}\text{P}$  labeling zone were located at the same distance from the plants in all the cuvettes, whereas the distance between the plant and the  $^{33}\text{P}$  labeling zone (2 cm wide, Fig. 1b) varied between 1.1 and 9.1 cm (Fig. 1a). Four cuvettes were prepared for each of the five distances between the plant and  $^{33}\text{P}$  labeling compartments. Before planting, the  $^{33}\text{P}$  labeled compartment was filled with the substrate labeled with  $^{33}\text{P}$  radioisotope solution (1 ml of aqueous  $^{33}\text{PO}_4^{3-}$  solution; carrier-free orthophosphate, Hartmann Analytic GmbH, Braunschweig Germany;  $4.14$  MBq  $\text{ml}^{-1}$ ). Forty-eight hours before harvest, 3 ml of aqueous solution of  $^{32}\text{PO}_4^{3-}$  ( $0.5$  MBq  $\text{ml}^{-1}$ ) were injected into the  $^{32}\text{P}$ -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  $^{32}\text{P}$  isotope into the labeling zone. The needle used for the  $^{32}\text{P}$  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  $^{33}\text{P}$  labeling zones. This resulted in a completely randomized design with 5 replicates with respect to all variables except  $^{33}\text{P}$  acquisition by plants, for which



**Fig. 1** Cultivation system (cuvette) used in this experiment. Dashed lines—500- $\mu\text{m}$  mesh, dotted lines—30- $\mu\text{m}$  mesh. Substrate labeled with  $^{33}\text{P}$  was added to the system as a 2-cm slice at different distances from the plant compartment (a). Plant compartment was separated from the root-free zone by a

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 (1970) and then washed in sterile water and germinated for 3 days on a moistened filter paper. Three seedlings were planted per plant compartment of each cuvette and grown there for 42 days.

Three AMF species, all isolated from a single field site in Switzerland (Jansa 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 as 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 (data not shown), 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

sandwich of three meshes (b). The  $^{32}\text{P}$  labeling solution was injected 48 h before the harvest in a compartment separated from the plants by two buffer zones (c) using a 15-cm needle with multiple openings (d) to ensure homogenous labeling of the substrate with the  $^{32}\text{P}$

substrate dry weight for *Glomus intraradices*, *Glomus claroideum* and *Gigaspora margarita*, respectively. Non-mycorrhizal cuvettes were mixed (1:20 v:v) with substrate, where non-mycorrhizal leek was grown for previous 10 months.

Plant compartments were watered every day and the root-free zone every other day. The plants received 50 ml cuvette<sup>-1</sup> week<sup>-1</sup> of a modified Hoagland nutrient solution (Hoagland and 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 measuring radioactivity in the shoots by a Geiger-Müller counter (see Table S1 in Online resource 1)

#### Harvest and measurements

Shoots, roots and substrate from the plant compartment were harvested after 6 weeks of growth. In the root-free zone, all substrate was collected from the

buffer zone 1 (Fig. 1c) and used to isolate the fungal hyphae from this zone. This was done by wet sieving through 500  $\mu\text{m}$  and 40  $\mu\text{m}$  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 by using a 8-mm cork borer or a spatula: 0.25 cm (buffer zone 1), 0.95 cm ( $^{32}\text{P}$  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). 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  $\text{HNO}_3$  (65%), made to 25 ml with distilled water and filtered through a paper filter (Whatman No 40). The concentrations of P and activity of both  $^{33}\text{P}$  and  $^{32}\text{P}$  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 (Packard, Zurich, Switzerland), 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  $^{33}\text{P}$  and  $^{32}\text{P}$  measurements with automatic quenching correction using single radioisotope solutions and different concentrations of a chemical quencher ( $\text{CCl}_4$ ). 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. 2003).

#### 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  $\mu\text{g P seed}^{-1}$ ) from total plant P content at harvest. Plant growth and P uptake benefits (Eqs. 1 and 2, respectively) were calculated according to Cavagnaro et al. (2003), using individual biomass and P uptake values of inoculated plants ( $M_b$  and  $M_p$ , respectively) and means of the biomass and P uptake values of the non-mycorrhizal plants ( $NM_b$  and  $NM_p$ , respectively; means of five replicates):

$$\text{Plant growth benefit(\%)} = \frac{M_b - NM_b}{NM_b} \times 100 \quad (1)$$

$$\text{P uptake benefit(\%)} = \frac{M_p - NM_p}{NM_p} \times 100 \quad (2)$$

Phosphorus use efficiency ( $\text{g mg}^{-1}$ ) was calculated by dividing the plant dry mass by the plant P content (Baon et al. 1993). Percentages of  $^{32}\text{P}$  and  $^{33}\text{P}$  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 each of the isotopes 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 efficiency of P uptake via AMF hyphae was calculated by dividing the  $^{32}\text{P}$  activity (kBq) recovered in the plants (shoot and roots combined) by the HLD ( $\text{m g}^{-1}$ ) measured in the  $^{32}\text{P}$  injection compartment. The statistics (ANOVA, multiple range tests) were calculated in Statgraphics Plus for Windows version 3.1.

#### Hyphal growth model

Measured HLD in the substrate at different distances from plants were further analyzed using the growth model for arbuscular mycorrhizal fungi by Schnepf et al. (2008a) 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 due to linear and nonlinear apical branching, tip–hyphae and tip–tip anastomoses and tip and hyphal death. These processes are responsible for the specific growth pattern of an AM fungus (Schnepf et al. 2008b). In particular, apical branching refers to a process where new hyphae are formed by tip splitting. Linear apical



branching refers to a branching pattern that is linearly proportional to the tip density while the nonlinear apical branching decreases with increasing tip density and ceases at a given maximal tip density (Schnepf et al. 2008a). For model equations see Online resource 2 (Equations S1–S4); the corresponding model parameters are described in Table 1. No direct measurements of these growth parameters were available. Therefore, our aim was to fit the fungal growth model to the measured HLD in order to obtain the set of parameters which gave the best fit.

The data on HLD were not collected primarily for this model exercise. This resulted in the fact that the number of data points was low with respect to model parameters and also that only data for one time point, i.e., at harvest, were available. In order to have the smallest possible number of parameters, we did not fit the full model of Schnepf et al. (2008a) to the experimental data. We started with the simplest form of this model which only considers linear branching of hyphae, no anastomoses and a constant flux of tips across the root–fungus interface (i.e. 4 parameters to fit). It is also described in Schnepf et al. (2008a). Additional processes were only added when the fit could be improved. These processes were: nonlinear branching, tip-tip and tip-side anastomoses and

boundary proliferation (in total 6 were fitted in the most complicated case).

Computing the respective dimensionless parameters from these values as described in Schnepf et al. (2008a) (see also Online resource 2; Equations S5–S9) allowed us to interpret different growth patterns for the different fungal species. The dimensionless parameters represent the relative importance of the different processes included in the model compared with each other and are described in Table 1.

The model was run for a simulation time of 42 days (see Section “Plants and AMF”). Following assumptions and preparatory steps were made before the model was fitted to the measured HLD (see also Online resource 2; Equations S10–S12):

- 1) The model requires HLD of the AM fungus only. Therefore, HLD in the substrate with non-mycorrhizal plants was subtracted from the HLD of each species. Units were converted from  $\text{m g}^{-1}$  to  $\text{m cm}^{-3}$  by using the measured soil bulk density of  $1.3 \text{ g cm}^{-3}$ .
- 2) Each mean of HLD was calculated from 5 replicates and the standard error of the mean (ste) obtained as  $\text{std}/\sqrt{m}$  (with  $\text{std}$  = standard deviation and  $m$  = number of replicates, here 5).

**Table 1** Parameters (a) and dimensionless parameter values (b) of the fitted hyphal growth model

Symbol	Name	Units	<i>G. int</i>	<i>G. clar</i>	<i>Gi. m</i>
(a)					
$v$	elongation rate constant	$\text{cm d}^{-1}$	0.2296	0.2008	0.0437
$b$	net apical branching rate constant	$\text{d}^{-1}$	0.4872	8.8664	0.3871
$d$	hyphal death rate constant	$\text{d}^{-1}$	0.0006	0.0566	0.3643
$f$	tip reduction rate due to nonlinear branching and tip-tip anastomosis	$\text{cm}^3 \text{ d}^{-1}$	$2.78 \times 10^{-4}$	$8.21 \times 10^{-2}$	$1.10 \times 10^{-4}$
$a_{np}$	tip-side anastomosis rate constant	$\text{cm}^3 \text{ m}^{-1} \text{ d}^{-1}$	0.0981	–	–
$a$	root-fungus boundary proliferation rate constant	$\text{m cm}^{-3} \text{ d}^{-1}$	0.0656	–	–
$\rho_{0,b}$	initial length density at root-fungus boundary	$\text{m cm}^{-3}$	–	8.1573	29.5434
(b)					
$\delta$	relative importance of hyphal death with respect to apical branching	dimensionless	0.0012	0.0064	0.9412
$\alpha_{np}$	relative importance of tip-hypha anastomosis with respect to branching	dimensionless	1.9073	–	–
$\beta$	relative importance of nonlinearity in apical branching and tip-tip anastomosis in relation to elongation	dimensionless	1.1487	302.56	7.4319
$\psi$	dimensionless proliferation at boundary	dimensionless	$8.5 \times 10^{-6}$	–	–
$\xi$	dimensionless initial tip density at boundary	dimensionless	0.0142	0.0070	0.9412

*G. int* *Glomus intraradices*, *G. clar* *Glomus claroideum*, *Gi. m* *Gigaspora margarita*

Both the HLD in the substrates with and without mycorrhizal species contributed to the overall error  $ste_T$  at each data point. It was calculated according to the Gaussian law of error propagation (Walpole et al. 2007).

- 3) The mesh separating plant and hyphal compartment was regarded as the root-fungus boundary. 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). The model was regarded acceptable, when  $RMSE < RMSE_{95\%}$  (the 95% RMSE confidence interval, Smith et al. (1997)). In this case, the simulated values are inside the 95% confidence interval of the measured values. The MATLAB function 'fminsearchbnd' was used in order to find the parameter set that minimizes the RMSE between measured and calculated hyphal length densities. This optimization algorithm uses the Nelder–Mead simplex method (Lagarias et al. 1998) and allows that bounds can be applied to the variables. It is obvious that the model fitting could be done using much more sophisticated fitting methods. However we feel that this would give and only enhance the false sense of security in the results. Essentially, in order to fit  $n$  number of independent parameters in the model at least  $n+$  number of independent experiments should be in principle conducted. However, doing these experiments would be in our opinion beyond the scope of this paper. The model used in this study is only for the purposes of data interpretation and hence all the conclusions drawn from the model should be taken

within this context and within the context of general way of using illustrative modeling.

## 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 across all the mycorrhizal treatments. 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 2; Fig. 2). The two *Glomus* treatments were more beneficial for the plant growth compared to the *Gigaspora* and the NM treatments (Table 2). Plant P uptake was also promoted by the three different mycorrhizal treatments (Table 2), 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 2).

Appearance of radioactivity in plant shoot measured by hand-held radio-monitor during the experiment (see Online resource 1; Table S1) showed faster elongation rate of the mycelium front for *Glomus intraradices* than for the other AMF species.

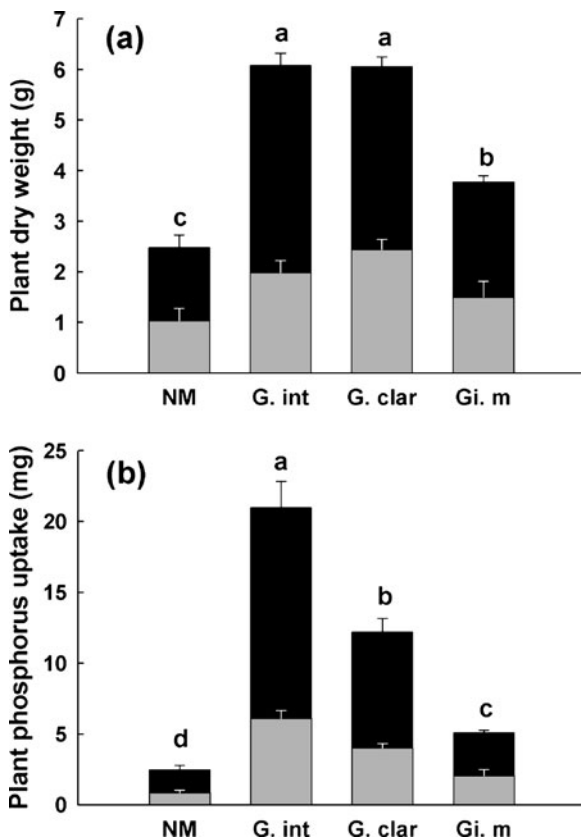
**Table 2** Growth and phosphorus (P) uptake benefits and P use efficiency of medic plants colonized by the different AMF species. Means of 5 replicates  $\pm$  standard deviations are shown. Different letters indicate significant differences among the means ( $p < 0.05$ )

	Plant growth benefit (%)	P uptake benefit (%)	P use efficiency (g mg <sup>-1</sup> )
NM	0 $\pm$ 18 c	0 $\pm$ 14 d	1.00 $\pm$ 0.10 a
<i>G. int</i>	145 $\pm$ 16 a	745 $\pm$ 77 a	0.30 $\pm$ 0.01 d
<i>G. clar</i>	144 $\pm$ 14 a	390 $\pm$ 33 b	0.50 $\pm$ 0.05 c
<i>Gi. m</i>	52 $\pm$ 10 b	105 $\pm$ 14 c	0.74 $\pm$ 0.04 b
ANOVA	F(3, 16)=117.3***	F(3, 16)=289.0***	F(3, 16)=120.4***

NM non-mycorrhizal control, *G. int* *Glomus intraradices*, *G. clar* *Glomus claroideum*, *Gi. m* *Gigaspora margarita*

\*\*\*  $p < 0.001$



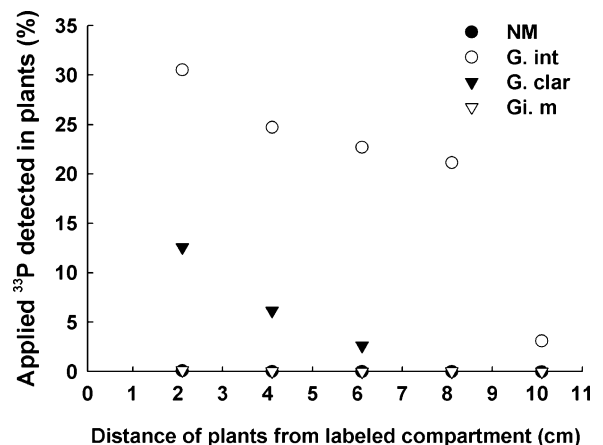


**Fig. 2** Combined shoot (black) and root (grey) biomass (a) and phosphorus uptake (b) of medic plants. Mean values of five replicate cuvettes  $\pm$  standard deviations are shown. Different letters indicate significant differences between the means according to the least significant difference-based 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*

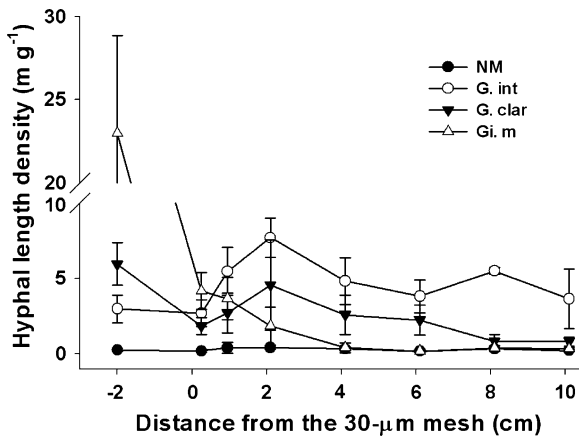
Furthermore, the radioactivity of  $^{33}\text{P}$  found in the medic plants (shoot and root combined) gives an indication of the maximal distance reached by the mycorrhizal hyphae when growing into the root-free zone (Fig. 3) within the duration of the experiment. The percentage of  $^{33}\text{P}$  transported to the NM plants from the labeled compartment nearest to the 30- $\mu\text{m}$  mesh was close to zero (0.09% of the applied  $^{33}\text{P}$  activity). In plants colonized by *Glomus intraradices*, significant amounts of  $^{33}\text{P}$  were transferred to the plants via the mycelium even from the greatest distance, 10.1 cm from the roots (Fig. 3). For *Glomus claroideum*, the  $^{33}\text{P}$  uptake took place at a maximal distance of 6.1 cm, while almost no  $^{33}\text{P}$  activity was detected in any of the plants colonized by *Gigaspora margarita* (Fig. 3).

The HLD was low and almost identical for the NM treatment at all sampling positions (gross mean of  $0.3 \pm 0.12 \text{ m g}^{-1}$ ; Fig. 4), with no significant differences between sampling positions ( $p = 0.20$ ). For plants 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  $7.7 \pm 1.3 \text{ m g}^{-1}$  in the root-free zone at a distance of 2.1 cm from the 30- $\mu\text{m}$  mesh. For the *Glomus claroideum* treatment, the maximal HLD was measured in the plant compartment ( $5.95 \pm 1.4 \text{ m g}^{-1}$ ) which was not statistically different from the HLD measured in the root-free zone 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 on average almost  $23 \text{ m g}^{-1}$  were recorded, towards to root-free zone (Fig. 4). Already at a distance of 2.1 cm from the roots, the measured HLD values were not significantly different from the NM treatment.

Transfer of  $^{32}\text{P}$  was significantly different between the three mycorrhizal treatments (Fig. 5a,  $p < 0.001$ ). Almost no  $^{32}\text{P}$  was found in the NM plants (0.08% of



**Fig. 3** Percentages of  $^{33}\text{P}$  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  $^{33}\text{P}$  placement and each fungal treatment. NM—non-mycorrhizal control, G. int—*Glomus intraradices*, G. clar—*Glomus claroideum*, Gi. m—*Gigaspora margarita*

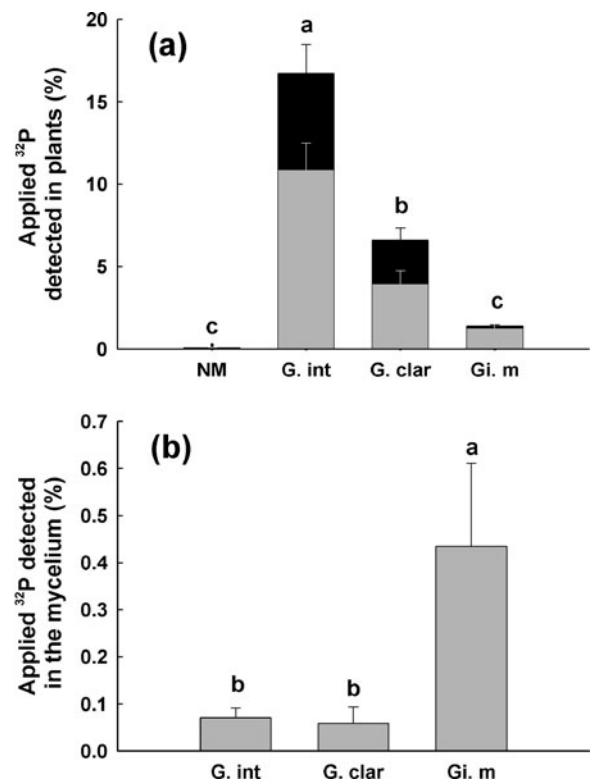


**Fig. 4** Length densities of the fungal hyphae in the plant compartment (distance -2 cm from the mesh) and in the root-free zone at increasing distances from the 30- $\mu$ m mesh. Values are given per unit substrate dry weight. Mean values of five replicates  $\pm$  standard deviations are shown. NM—non-mycorrhizal control, G. int—*Glomus intraradices*, G. clar—*Glomus claroideum*, Gi. m—*Gigaspora margarita*

the applied amount). The mycorrhizal P uptake pathway efficiency was different between the different AMF species ( $p=0.01$ ). The values reached 3.17, 2.94, and 0.38 kBq  $^{32}\text{P}$  g  $\text{m}^{-1}$  in 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 values of the mycorrhizal P uptake pathway efficiency. The percentage of  $^{32}\text{P}$  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. 5b), although the HLD values for all three AMF species in this compartment were comparable (Fig. 4).

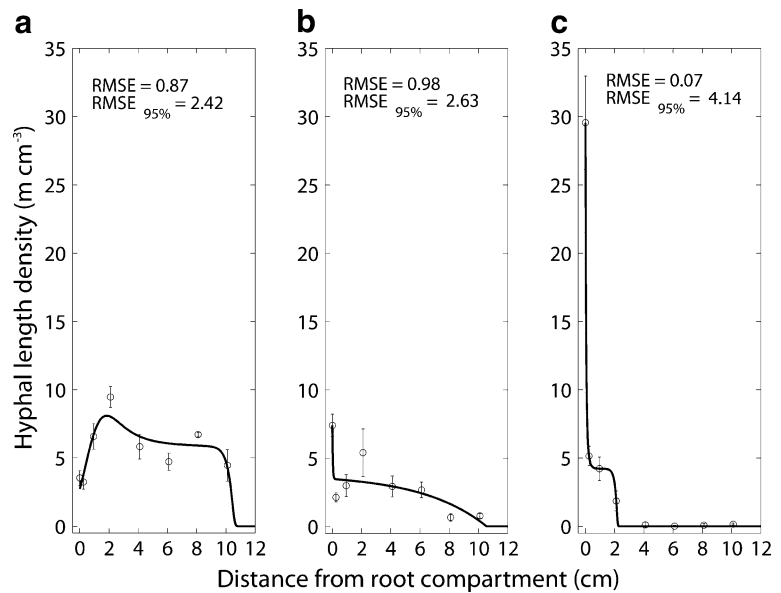
The numerical model for the AMF hyphal growth (Schnepf et al. 2008a) was fitted to the HLD of the three fungal species, starting with the simplest model (linear branching only, no anastomoses and no boundary proliferation) and adding additional mechanisms step by step only if required. This procedure 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 an objective choice about which value is

acceptable, we followed Smith et al. (1997) and thus compared the RMSE with the  $\text{RMSE}_{95\%}$ . The results showed that the model could be fitted to all the fungal species within the accuracy of the data provided (Fig. 6). This procedure resulted in a model with 5 parameters (see Table 1) for both *Glomus claroideum* and *Gigaspora margarita*. This model included, in addition to the simplest model with 4 parameters, also parameters describing nonlinear branching and tip-tip anastomoses. The best model for *Glomus intraradices* additionally includes tip-side anastomoses and hyphal boundary proliferation but has no initial hyphal length density at the root-fungus boundary. The number of model parameters



**Fig. 5** Percentages of  $^{32}\text{P}$  applied into the root-free zone and detected in the plants (a; shoot and roots combined), and in the mycelium extracted from the buffer zone 1 (b) at 48 h after the  $^{32}\text{P}$  labeling. Mean values of five replicates  $\pm$  standard deviations 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*

**Fig. 6** Fitting the measured hyphal length densities of AMF fungi in the substrate (open circles with bars indicating  $\pm$  standard errors) to the growth model (solid lines) for the mycorrhizal mycelium. Values of model root mean square error (RMSE) and its 95% confidence interval (RMSE<sub>95%</sub>) are shown. **(a)** *Glomus intraradices*, 6 model parameters; **(b)** *Glomus claroideum*, 5 model parameters; **(c)** *Gigaspora margarita*, 5 model parameters



for this species is 6 (see Table 1). These results should be viewed as one way of interpreting the data, but this is by no means the only way. The fit for the *Gigaspora margarita* was one of the best ones while the fitted curves for the other two are visually less accurate but they still satisfy our RMSE<sub>95%</sub> criteria (Fig. 6). The respective model parameter values as obtained from the fitting are shown in Table 1. The dimensionless parameters in the same table show the relative importance of the different growth mechanism for each fungal species. These results could be used to interpret the data in the following way:

- 1) *Glomus intraradices* scavenges locally for resources due to its pronounced nonlinear branching growth pattern. In addition, its equally pronounced tip-hypha anastomoses growth pattern results in the creation of an interconnected mycelium that facilitated resource transport within the mycelium.
- 2) *Glomus claroideum* mycelium network growth is strongly dominated by nonlinearity in apical branching. One way of interpreting this result would be to say 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 compart-

ment (i.e. the 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.

## Discussion

### Root colonization

Reasonable levels of root colonization (above 40%) were observed for the three AMF treatments, whereas no colonization was observed in the NM treatment. These (rather high) colonization levels are probably due to choice of the host plant species (e.g., compare with Li et al. 2008), as well as due to high quality of mycorrhizal inocula. Although there is no general consensus about how much colonization is needed to achieve maximum benefits to the plants for 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, stimulation of plant growth and plant P uptake responses to inoculation

are compared (Abbott and 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

Plant growth benefits were high for the two *Glomus* species (145% on average) and somehow lower for *Gigaspora margarita* (52%). Other studies have reported similar plant growth benefits for *Glomus intraradices* and *Glomus claroideum* (Jansa et al. 2008; Smith et al. 2004) for *Medicago truncatula* colonized to a similar degree as in this study. In previous 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; Li et al. 2008; Smith et al. 2004).

Unlike the plant growth response, the two *Glomus* species were significantly different in term of P uptake benefit with a value reaching 745% for *Glomus intraradices* and 390% for *Glomus claroideum*. These are high values, comparable with the P benefits in very low P soils (e.g. Jakobsen et al. 1992b), but can also be explained by access of the fungi into soil volume which was not accessible to the NM roots due to spatial confinement. This might have led to overestimation of mycorrhizal benefits as discussed by Smith et al. (2000).

### Mycorrhizal P 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  $^{33}\text{P}$  activity detected in plants colonized by the AMF 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 thus confirmed those

obtained by a previous study for the two *Glomus* species (Jansa et al. 2005) where a similar experimental design—though with a different host plant—was employed. To our knowledge, it is the first time that such an 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, 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 the generally thick *Gigaspora* hyphae (diameter estimated by microscopy was about 8  $\mu\text{m}$  on average versus 4–5  $\mu\text{m}$  for the *Glomus* species).

In order to further characterize the implication of the mycorrhizal hyphae development in the P acquisition, the HLD has been measured for the three species in the different compartments of the root-free zone and in the plant compartment. The results generally support the  $^{33}\text{P}$  uptake data in a similar manner as established previously (Schweiger et al. 1999; Jansa et al. 2005). In the plant compartment, the inoculation with *Gigaspora margarita* has produced large amounts of mycelium with a value reaching on average 23  $\text{m g}^{-1}$ . This result is consistent with many other studies (e.g. Hart and Reader 2002b) that have shown that members of this genus usually colonize intensively the substrate close to the roots but no specific distance had ever been mentioned (although e.g. Smith et al. (2004) observed no detectable proliferation of *Gigaspora rosea* beyond a mesh barrier). 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  $3 \times 10^4$  m of hyphae produced, followed by *Glomus intraradices* and *Glomus claroideum* with  $2 \times 10^4$  m and  $1.45 \times 10^4$  m, respectively. These numbers indicate that it is more the maximal distance from roots reached the hyphae than the total mycelium production that explains the diversity observed in the phosphorus acquisition of the mycorrhizal plants in this experiment.

Modeling of HLD indicates important differences between the three AMF used in this study. This is the first study indicating substantial differences in mechanisms of the hyphal network formation

between species of the same genus *Glomus*, and not only between different AMF genera. Precautions should be, however, 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 points (8) being inferior to the number of full model parameters (11). Future study will need even more spatially detailed approach as well as sequential harvests at different times and some direct estimations 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. Particularly important is the case of *Glomus intraradices*, which seems to form denser hyphal networks at a certain distance from the roots than close to the roots. Like in other studies (Jakobsen et al. 1992b; Jansa et al. 2003), 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. Similarities to previous results in fitting this fungal growth model to experimental data could be found. The dominant mechanism found for *Glomus claroideum*, nonlinear branching/tip-tip anastomosis, was also found to be dominant (Schnepf et al. 2008a) for the *Glomus* sp. used by Jakobsen et al. (1992b). *Gigaspora margarita* was characterized by extensive production of hyphal tips at the root-fungus interface together with high hyphal turnover rate. Schnepf et al. (2008a) found this to be the case for *Scutellospora calospora* (using data from Jakobsen et al. (1992b)). It needs yet to be proven whether the mechanisms identified as key differences between the different AMF in the model are correctly assigned or whether there are additional important mechanisms such as proliferation of thin and short-living hyphal structures like those described by Bago et al. (1998).

#### Efficiency of the P uptake via AMF hyphae

Six weeks after the start of the experiment, it was assumed that all the three mycorrhizal treatments would have produced extraradical mycelium at least

at a small distance from the roots. The  $^{32}\text{P}$  labeling was therefore employed to assess the efficiency of the mycorrhizal plant P uptake pathway. The  $^{32}\text{P}$  activity found in the plants indicates an important diversity with regards to this feature while the HLD in the  $^{32}\text{P}$ -labeled compartment were in the same range for all the three species. The different values of the  $^{32}\text{P}$  transfer by the different AMF to the plants seem to indicate that the HLD and the P uptake via the mycorrhizal pathway may well be comparable between closely related species but that they are possibly very different for phylogenetically distant species (Smith et al. 2004; Jansa et al. 2005). Rare information on the functional diversity within AMF species further supports this notion (Jansa et al. 2005; Munkvold et al. 2004). Other studies established that functional diversity may indeed be phylogenetically structured (Hart and Reader 2002a, b) and this could be one of the reasons for the plants to associate with different AMF species at the same time (Maherali and Klironomos 2007). Different reasons could explain the low  $^{32}\text{P}$  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 (e. g. having a high  $K_m$  value). We could also speculate that decent amounts of the  $^{32}\text{P}$  had been taken up by the hyphae but that its low transfer to the plants was due to a delay of its delivery at the root-arbuscules interface. To further elucidate this issue, mycelium of the buffer zone 1 (see Fig. 1) has been harvested and further processed to determine its  $^{32}\text{P}$  activity and P content at the time of harvest. The result presented in Fig. 5b shows a significantly higher radioactivity in the mycelium from 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 (Boddington and Dodd 1998; Jakobsen et al. 1992a), where it has been suggested that AMF species from the Gigasporaceae might store significant amounts of their acquired 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 and 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  $^{32}\text{P}$  in the AMF extraradical hyphae 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 its mycelium and for spore formation. This argument is further supported by the data shown in Figs. 2 and 5a. While biomass and P are distributed between roots and shoot in the same proportion for the three mycorrhizal treatments, the ratio for  $^{32}\text{P}$  activity in roots to the same in the shoots is significantly higher for *Gigaspora margarita* as compared to the two *Glomus* treatments ( $p < 0.001$ ). It is probable that most of the  $^{32}\text{P}$  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 P in roots colonized by *Gigaspora margarita* was indeed within the fungal structures (Solaiman and Saito 2001).

#### P uptake strategies of the mycorrhizal plants

$^{33}\text{P}$  labeling indicated that in the case of the *Glomus* species, the P taken up by the plants via the mycorrhizal pathway may originate from several cm away from the roots while for *Gigaspora margarita*-colonized plants it clearly comes from a limited volume of substrate close to the roots. Moreover, modeling results do relate to measured P uptake in this study. Indeed, we found that the fungal species characterized by tip-hypha anastomoses, *G. intraradices*, took up P from up to 10 cm distance. A typical feature of tip-hypha anastomosis is a local peak near the front of the colony, and this peak occurs at 10 cm distance in this study. When the effect of nonlinear branching/tip-tip anastomoses is large, as found for *G. claroideum*, the production of new tips is reduced when the tip density is large and HLD is reduced at the front of the colony. Therefore, it makes sense that measured P uptake did not extend to the edge of the colony. The high hyphal turnover, as postulated for *G. margarita* based on the numerical modeling, explains the low HLD at larger distances from the root surface and the corresponding lack of  $^{33}\text{P}$  uptake. These different spatial abilities to acquire P might help explain empirical evidence that colonization of roots by multiple AMF species can be more

beneficial than colonization by one species only (Jansa et al. 2008).

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 temporarily immobilized in the fungal mycelium before being delivered to the 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 *Glomus claroideum*, which proved to be less efficient in P acquisition than *G. intraradices*, probably required less carbon from the plants than *Glomus intraradices* (as could be inferred from the higher PUE in plants colonized by *G. claroideum* as compared to those colonized by *G. intraradices*), the levels of growth promotion by both of the *Glomus* species were similar.

The results obtained here contribute to a better understanding of the functional diversity observed between AMF species with regards to the P acquisition from the soil and transfer to the plants, and generates further hypotheses to be tested in the future. Among the relevant parameters playing a role are 1) ability of AMF to colonize the substrate (and probably only to a lesser extent the roots) expressed in term of rate and extent (maximal distance reached from the roots) of substrate colonization; 2) efficiency of the plant P uptake via AMF hyphae (efficiency of absorption of the inorganic phosphates by the hyphae from the soil solution, transport within the mycorrhizal mycelium and delivery at the root-arbuscule interface), and 3) carbon cost of the mycorrhizal symbiosis (requirements for carbohydrates fixed by the plant photosynthesis for growth and maintenance of the associated AMF). Indeed, the differences in magnitude and rate of carbon fluxes between plant and AMF appear to be of paramount importance for explaining differences in growth promotion of plants due to mycorrhizal establishment. Furthermore, numerical modeling as employed in this research showed a novel approach to generate testable hypotheses on the mechanisms underlying the observed patterns in hyphal network dynamics and P acquisition by the AMF hyphae from the soil.

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