

# Symbiont identity matters: carbon and phosphorus fluxes between *Medicago truncatula* and different arbuscular mycorrhizal fungi

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**Abstract** Many studies have scrutinized the nutritional benefits of arbuscular mycorrhizal associations to their host plants, while the carbon (C) balance of the symbiosis has often been neglected. Here, we present quantification of both the C costs and the phosphorus (P) uptake benefits of mycorrhizal association between barrel medic (*Medicago*

*truncatula*) and three arbuscular mycorrhizal fungal species, namely *Glomus intraradices*, *Glomus claroideum*, and *Gigaspora margarita*. Plant growth, P uptake and C allocation were assessed 7 weeks after sowing by comparing inoculated plants with their non-mycorrhizal counterparts, supplemented with different amounts of P. Isotope tracing ( $^{33}\text{P}$  and  $^{13}\text{C}$ ) was used to quantify both the mycorrhizal benefits and the costs, respectively. *G. intraradices* supported greatest plant P acquisition and incurred high C costs, which lead to similar plant growth benefits as inoculation with *G. claroideum*, which was less efficient in supporting plant P acquisition, but also required less C. *G. margarita* imposed large C requirement on the host plant and provided negligible P uptake benefits. However, it did not significantly reduce plant growth due to sink strength stimulation of plant photosynthesis. A simple experimental system such as the one established here should allow quantification of mycorrhizal costs and benefits routinely on a large number of experimental units. This is necessary for rapid progress in assessment of C fluxes between the plants and different mycorrhizal fungi or fungal communities, and for understanding the dynamics between mutualism and parasitism in mycorrhizal symbioses.

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## Introduction

In arbuscular mycorrhizal (AM) symbiosis, mineral nutrients such as phosphorus (P), zinc, copper, and/or nitrogen are gathered from the soil by the fungal hyphae

and transferred to the roots, while the plant supplies reduced carbon (C) compounds to the fungus (Smith and Read 2008; Parniske 2008). Since mineral nutrient supply is one of the common constraints of plant growth and reproduction in many ecosystems, and the heterotrophic AM fungi are provided with exclusive access to easily metabolizable C compounds, this association often ends up as a mutualistic symbiosis, i.e., beneficial for both involved parties (Jones and Smith 2004; Smith and Smith 1996). The exact outcome of the symbiosis for the plants (i.e., the extent of plant growth promotion, nutritional improvements, etc.) depends, however, on the identity of both plant and fungus, as well as on the properties of the environment such as climate, nutrient status of soil, light availability, and others (Jones and Smith 2004; Li et al. 2008). In some situations, the benefit-to-cost ratio for some host plants may instead be reduced as a consequence of association with certain AM fungi, qualifying the association as parasitic (Graham and Abbott 2000; Graham and Eissenstat 1998; Li et al. 2008).

In contrast to the detailed knowledge gathered in the past on the diversity of P uptake strategies of and benefits provided to the plants by different AM fungi (Jakobsen et al. 1992a; Jansa et al. 2005; Munkvold et al. 2004; Smith et al. 2004; Thonar et al. 2011), little is known about the C costs of AM symbiosis for the host plant. Up to 20% of the carbon fixed in plant photosynthesis has been reported to be allocated to the AM fungus *Glomus fasciculatum* colonizing cucumber plants, based on  $^{14}\text{CO}_2$  labeling (Jakobsen and Rosendahl 1990). Other studies based on the same methodology reported allocations between 4% and 16% of plant C to different AM fungal species (Bryla and Eissenstat 2005; Grimoldi et al. 2006; Jakobsen and Rosendahl 1990; Kaschuk et al. 2009; Paul and Kucey 1981). Few studies have used  $^{13}\text{CO}_2$  labeling to track the fate of C from the shoot to the rhizosphere (Gavito and Olsson 2003; Olsson and Johnson 2005; Olsson et al. 2005). However, these were generally restricted to measurement of allocation of C to standing biomass of the AM fungi (using  $^{13}\text{C}$ -allocation to signature fatty acids) and thus did not consider a large fraction of C diverted towards the AM fungi, i.e., the C respired by the fungi on a short term (Johnson et al. 2002; Grimoldi et al. 2006). Thus far, only one study using  $^{13}\text{CO}_2$  labeling has succeeded in the enumeration of full carbon balance of a plant (*Lolium perenne*) colonized by *Glomus hoi* (Grimoldi et al. 2006). The estimate of C flow into the AM fungal growth and respiration was in this case below 8% of the daily gross photosynthesis.

To achieve a better understanding of mycorrhizal functioning, there is a need to experimentally measure both the symbiotic C costs and the growth and/or P uptake benefits of mycorrhizal plants in a simple system, allowing large replication within the experiments. This is exempli-

fied by the fact that little information is currently available on the C costs of plants associated with different AM fungal species/isolates. To our knowledge, only one study thus far explicitly addressed the variability of C costs between different AM fungal taxa while measuring the mycorrhizal P uptake benefits to the host plant at the same time (Pearson and Jakobsen 1993). This study clearly showed that colonization of cucumber roots by *Scutellospora calospora* resulted in larger C drain belowground as compared with two *Glomus* species, while also providing the least P uptake benefits to the plants. The absolute C allocation to the fungi was in all these cases lower than the 20% of plant C reported elsewhere (Jakobsen and Rosendahl 1990). However, large differences in plant growth and P nutrition between the different AM fungal treatments probably biased some of the comparisons. Similar constraints (different biomass and P content between plants colonized by different AM fungal species and the non-mycorrhizal plants) and lack of isotopic C labeling also limited precise assessment of the C costs in a more recent study (Thonar et al. 2011). Concurrent measurements of both mycorrhizal C costs and growth/nutritional benefits on the same plant will thus constitute a crucial step towards the understanding of the functioning of mycorrhizal communities that include more than one AM fungal species within the root system of a single plant, which is a general case rather than an exception (Jansa et al. 2003; Merryweather and Fitter 1998). This also appears important as topics like the preferential allocation of C by the plants to different fungi within the same root system are being considered (Bever et al. 2009) and conjectures about C costs of mycorrhizal communities under field conditions are being made (Verbruggen and Kiers 2010).

Here, we designed a simple experimental setup to quantify both the costs and benefits of mycorrhizal symbiosis on the same experimental units. For this, we used a model plant *Medicago truncatula* Gaertn. (medic), inoculated or not with one of three different AM fungal species.  $^{13}\text{C}$  and  $^{33}\text{P}$  were used as tracers to track movement of C and P through the experimental system. The following objectives shaped this experimental work:

1. To quantify mycorrhizal costs to the plants incurred by different AM fungal species, using two approaches: first, by measuring belowground respiration levels of mycorrhizal and non-mycorrhizal plants of approximately the same size, and second, by determining allocation of recently assimilated C (applied as a  $^{13}\text{CO}_2$  pulse) to shoot, roots, substrate (both rooted and root-free), and belowground respiration.
2. To quantify mycorrhizal benefits to the plants due to establishment of mycorrhizal symbiosis with different AM fungal species in terms of growth improvement, P acquisition, and transport of  $^{33}\text{P}$  from a root-free soil patch.

## Materials and methods

### Biological materials

Seeds of *M. truncatula* Gaertn. (medic) cv. Jemalong J5 were surface-sterilized in concentrated (97 %) sulfuric acid for 12 min (Massoumou et al. 2007) and then repeatedly washed in sterile tap water, thereby achieving seed surface sterility and synchronous germination. The seeds were then germinated on moist filter paper at 24°C for 3 days.

Inocula of three AM fungal species (*Glomus intraradices*, *Glomus claroideum*, and *Gigaspora margarita*; Table 1), previously isolated from a single field site in Tänikon, Switzerland (Jansa et al. 2002), were produced in pot cultures with leek (*Allium porrum* L.) cv. Du Bouchet as a host plant for 10 months under glasshouse conditions (26/20°C day/night, respectively, 16 h photoperiod with a minimum of 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Inoculation densities (Table 1) were adjusted based on previous infectivity assay (data not shown), to obtain approximately 50% root length colonized by the different AM fungal species at the end of the experiment. As such, small amounts of the *Glomus* spp. inocula were mixed in the substrate to achieve the desired spore densities quoted in Table 1. Spores of *Gigaspora* were first sieve-washed from large amount of fungal inoculum and then mixed into the substrate.

### Experimental setup and management

Five treatments were considered as follows: non-mycorrhizal control, non-mycorrhizal control with additional P supply (30 mg orthophosphate  $\text{pot}^{-1}$ ), and three single AM fungal species treatments. Five replicate pots were established for each treatment in a fully randomized design.

Plants were grown in 700-ml pots sterilized with 100% ethanol. Each pot was first layered with 250 g of heat-sterilized gravel (5–8 mm) at the bottom, which provided efficient drainage. This layer was covered with 700 g of substrate. The substrate consisted of sterilized soil from Tänikon, Switzerland (texture, sand 49%; silt, 32%; clay, 16%; soil organic matter, 3%), coarse quartz sand (grain diameter, 0.7–1.2 mm), and fine quartz soil (grain diameter, 0.08–0.2 mm) mixed in a ratio of 1:3:1 (v/v/v). The soil was

$\gamma$ -irradiated at Studer Hard, Däniken, Switzerland ( $^{60}\text{Co}$  source, 25–75 kGy). All sands were sterilized by heating at 120°C for 2 days. Substrate mixture was prepared from dry components, to which 3% water (v/v) was added upon mixing to allow for better homogenization. In the mycorrhizal treatments, the substrate was thoroughly mixed with the calculated amount of mycorrhizal inoculum in order to achieve the desired number of spores per pot (Table 1). The non-mycorrhizal treatments received 10 g  $\text{pot}^{-1}$  of substrate from pots in which non-mycorrhizal leek was previously grown for 10 months (“mock-inoculum”).

A root-free compartment consisting of a plastic vial filled with 36 g of radiolabeled substrate (carrier-free  $^{33}\text{P}$ -orthophosphoric acid, Hartmann Analytic GmbH, Braunschweig, Germany; 1.335 MBq  $\text{vial}^{-1}$ ) was inserted horizontally into each pot (Fig. 1). Nylon mesh (30  $\mu\text{m}$ , Sefar, Thal, Switzerland) sealed the opening of the vial, allowing the fungal hyphae to penetrate into the vials while preventing root access (Fig. 1). One pre-germinated medic seed was planted in each pot. The 25 pots were randomly positioned in a growth chamber PGV 36 (Conviron, Winnipeg, Canada) under 22/18°C and 75/90% relative air humidity (day/night, respectively). Light was provided as a combination of fluorescent and incandescent light sources, its intensity was 330  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Photoperiod was 14 h per day.

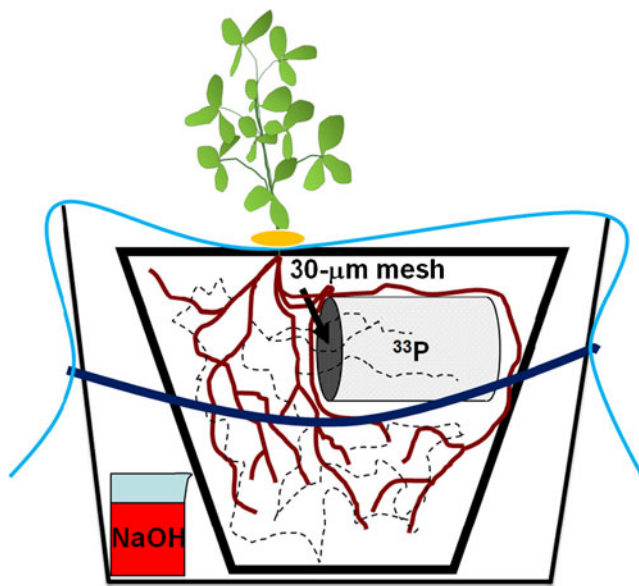
The plants were watered daily with distilled water to approximately 70% water holding capacity. Each pot received 100 ml of P-free Hoagland nutrient solution (Hoagland and Arnon 1950) weekly. The non-mycorrhizal+P treatment was provided, on a weekly basis, with 6 mg of P  $\text{pot}^{-1}$  (20 ml aqueous solution of  $\text{NaH}_2\text{PO}_4$ ), added to the pots over the first 5 weeks of growth, amounting to a total addition of 30 mg P per pot. The plants were grown for 44 days in the growth chamber before the  $\text{CO}_2$  labeling.

No severe nutrient deficiency symptoms (apart from the size differences due to P fertilization and/or mycorrhizal inoculation) were observed on the plants throughout the experiment. No nodules were observed on the roots upon harvest, confirming that the AM fungal inocula did not contain any significant loads of compatible rhizobial symbionts, which could potentially confound the result of this study.

**Table 1** Spore densities in the mycorrhizal inoculum and amounts of spores applied to the pots

Arbuscular mycorrhizal fungal species	BEG <sup>a</sup> accession number	Spores in inoculum ( $\text{g}^{-1}$ dry weight)	Spores applied per pot
<i>Glomus intraradices</i>	158	194	385
<i>Glomus claroideum</i>	155	126	497
<i>Gigaspora margarita</i>	152	93	11,200

<sup>a</sup> The International Bank for the Glomeromycota, [www.kent.ac.uk/bio/beg](http://www.kent.ac.uk/bio/beg)



**Fig. 1** Experimental setup. Plants were grown in pots with a root-free radiolabeled ( $^{33}\text{P}$ ) zone, separated from the root compartment by a 30- $\mu\text{m}$  mesh. This labeled zone was accessible only to the mycorrhizal hyphae (dashed lines) and not to the roots (solid lines). As of the pulse labeling with  $^{13}\text{CO}_2$ , the pots were kept in larger containers with NaOH traps, with their belowground compartment sealed off with an air-tight plastic film fastened around the containers with a rubber band (thick solid band). The film was sealed around the shoot basements with a plasticine. Drawing is not to scale

### $^{13}\text{CO}_2$ pulse labeling

Before labeling, the belowground part of the pots was hermetically sealed off (Fig. 1) by using polyethylene food-wrapping film (Saran Premium Wrap Nr 10, Migros, Zurich, Switzerland) and plasticine (non-hardening modeling clay, Jovi-Plastilina, ENasco, Atkinson WI, USA). The plants were moved to a separate growth chamber for  $^{13}\text{C}$  labeling. All 25 sealed pots were placed in an air-tight Plexiglas labeling chamber ( $0.35\text{ m}^3$ ) equipped with a fan ensuring constant mixing of air, and through which air could be pumped. Temperature, humidity and  $\text{CO}_2$  concentration in the chamber atmosphere were monitored throughout the labeling procedure (open path  $\text{CO}_2/\text{H}_2\text{O}$  gas analyzer LI-840, Lincoln, NE, USA; 0.1 Hz).  $\text{CO}_2$  in the labeling chamber was scrubbed down to  $290\ \mu\text{mol mol}^{-1}$  prior to labeling, by pumping the incoming chamber air through 4 l of 20% KOH solution. Pumping then stopped, the chamber was darkened and  $^{13}\text{CO}_2$  was released in the chamber over 5 min by adding 20% phosphoric acid through a septum in the labeling chamber onto >99 %  $^{13}\text{C}$ -enriched calcium carbonate powder (5 g, Sigma-Aldrich, Buchs, Switzerland). After labeling, the system was left to equilibrate in the dark for additional 10 min,

during which  $\text{CO}_2$  concentration reached  $604\ \mu\text{mol mol}^{-1}$ . Subsequently, lights were turned on and the plants were left to acquire the labeled  $\text{CO}_2$  for 1 h, during which the measured  $\text{CO}_2$  concentration dropped to  $570\ \mu\text{mol mol}^{-1}$ . Following the  $\text{CO}_2$  labeling, the pots were moved back to the growth chamber used for plant cultivation, and maintained for the next 5 days under the initial growth conditions.

### Belowground $\text{CO}_2$ efflux measurements

Following the  $^{13}\text{C}$  labeling,  $\text{CO}_2$  released from the belowground compartment was trapped over 5 days using alkaline traps ( $\text{CO}_2$ -free 1 M hydroxide solution, Titrisol, VWR, Dietikon, Switzerland; 35 ml each) previously placed in the sealed belowground compartment (Fig. 1). By using glucose (either  $^{12}\text{C}$  or  $^{13}\text{C}$ -glucose) additions to soil in such sealed pots we had previously confirmed that the seal was tight and that these traps absorbed approximately 90% of the  $\text{CO}_2$  produced by soil respiration from the atmosphere within the belowground compartments (see Electronic supplementary materials, Fig. S1). The traps were renewed daily and were processed according to Harris et al. (1997). Briefly, trap solution was transferred into 250-ml argon-purged Erlenmeyer flasks. Then, 19 ml of 1 M  $\text{SrCl}_2$  was added. The amount of  $\text{CO}_2$  collected in the traps was revealed after titration of the remaining hydroxide ions with 1 M HCl solution (Alef 1995). For the titration, phenolphthalein (0.1% in ethanol) was used as pH indicator. Following titration, the samples were centrifuged in 50-ml vials, the  $\text{SrCO}_3$  sediment washed three times with distilled water, transferred into 2-ml vials, and dried in a vacuum centrifuge (Heto CR-1, LAS Laborapparate AG, Appenzell, Switzerland). Approximately 4 mg of the  $\text{SrCO}_3$  (equivalent to approximately 325  $\mu\text{g C}$ ) was weighed together with 10 mg  $\text{V}_2\text{O}_5$  into tin capsules ( $5 \times 9\text{ mm}$ , 0.15 ml; Brechbühler, Schlieren, Switzerland), and the C-isotopic composition of the carbonate was analyzed with an elemental analyzer (Flash EA 1112 Series, Thermo Scientific, Rhodano, Italy) coupled to an isotope ratio mass spectrometer (Delta<sup>plus</sup>XP, Finnigan MAT, Bremen, Germany) via a six-port valve (Brooks et al. 2003) and a ConFlo III (Werner et al. 1999). To monitor potential memory effects, a urea sample with a natural abundance isotopic signature was measured before each laboratory standard. The isotopic signature of  $\text{CO}_2$  was expressed as the relative difference of its isotope abundance ratio relative to that of the Vienna

<sup>1</sup> Since the open path  $\text{CO}_2/\text{H}_2\text{O}$  analyzer does not measure  $^{12}\text{CO}_2$  and  $^{13}\text{CO}_2$  with the same efficiency, this number is only providing a crude estimate of the  $\text{CO}_2$  concentration in the chamber atmosphere, and was not used in any calculations.



PeeDee Belemnite (V-PDB) international standard. This relative difference is defined as:

$$\delta^{13}\text{C} = \frac{(^{13}\text{C}/^{12}\text{C})_{\text{Sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{V-PDB}}} - 1 \quad (1)$$

with the  $^{13}\text{C}/^{12}\text{C}$  ratio of V-PDB of 0.0111802 (Werner and Brand 2001). The long-term precision (~1.5 years) of the laboratory quality control standard (caffeine) for  $\delta^{13}\text{C}$  was 0.09‰.

#### Plant and substrate sample collection

Plants were harvested 49 days after transplanting the seedlings into the pots. Shoots were cut just above the substrate and roots were washed from the substrate with cold tap water. Fresh weight of both shoots and roots was recorded. Then, shoots were dried for 48 h at 75°C and weighed.

Root architecture parameters were assessed by scanning (WinRhizo version 2003b, Régent Instruments Inc., Canada, see Table S3 in the Electronic supplementary materials for details). Then, the root systems were cut into 1-cm fragments and thoroughly mixed to allow the recovery of representative root samples. The cut roots were divided into three portions. (1) Approximately 40% were stored at -20°C for DNA extraction and molecular quantification of AM fungal development (see Tables S1 and S2 in the Electronic supplementary materials for details). (2) Aliquots totaling approximately 30% were dried (48 h at 75°C) to obtain a gravimetric estimation of the dry weight. Furthermore, these aliquots were used for P and  $^{33}\text{P}$  analyses as well as to assess the  $\delta^{13}\text{C}$ . (3) Approximately 30% of the roots were transferred to plastic vials and frozen at -20°C for microscopic assessment of root colonization by the AM fungi (see Fig. S2 in the Electronic supplementary materials for details).

Approximately 40 g of the substrate from each pot and the radiolabeled vials were immediately stored at -20°C, preventing any biological degradation. These samples were later used for mycelium length density assessment, molecular quantification of AM fungal development, and  $\delta^{13}\text{C}$  measurements following freeze-drying.

#### Phosphorus and $^{33}\text{P}$ in plant biomass

Approximately 1 g of dried shoots or 0.2 g of dried roots were weighed into ceramic crucibles and covered with aluminum foil to prevent ashes from flying. Samples were incinerated in a muffle furnace (550°C for 10 h). After the ashes cooled down to room temperature, 2 ml 65% nitric acid was added to each sample and the samples briefly

heated to 250°C on a hot plate. After the samples cooled down again, Nanopure water was used to transfer the solutions containing all inorganic P to volumetric flasks through ashless filter paper. The extracts were diluted to 25 ml with Nanopure water. Phosphorus concentration in the extracts was measured in the samples diluted 100×, using the malachite green method (Ohno and Zibilske 1991).

To estimate  $^{33}\text{P}$  activity in the extracts, 2-ml aliquots were transferred to 25-ml scintillation vials and 10 ml of scintillation cocktail for acid samples was added (Ultima Gold AB, Packard, Goningen, the Netherlands). Radioactivity was measured on a  $\beta$ -counter (Packard 2500 TR, Packard, Goningen, the Netherlands) at an energy window between 2 and 220 keV for 10 min or until 50,000 counts were registered, while automatically subtracting background (5.2 %  $\text{HNO}_3$  solution) activity.

#### Carbon content and $\delta^{13}\text{C}$

Shoots, roots and substrate samples (from both the root and the root-free compartments) were dried (48 h at 75°C) and ground to fine powder before  $\delta^{13}\text{C}$  measurements (described above) and measurements of C concentrations were performed, the latter being carried out using elemental analyzer Flash EA 1112 (Thermo Electron Corporation, Waltham MA, USA).

#### Calculations and statistical analyses

Total root dry weights were calculated using the complete root system fresh weights collected at harvest and the ratios of dry to fresh weight of the root aliquots subjected to drying. Phosphorus contents of the roots and shoots were calculated from P concentrations and the dry weight data obtained gravimetrically. The proportion of  $^{33}\text{P}$  transferred to the was calculated using the ratio between measured  $^{33}\text{P}$  activities in the roots plus shoots and the amount of initial  $^{33}\text{P}$  amounts applied per pot. These values were corrected for the radioactive decay between the measurements of the labeling solution and of the samples after harvest.

Excess  $^{13}\text{C}$ , i.e., the amount of  $^{13}\text{C}$  originating from the  $^{13}\text{CO}_2$  introduced to the system upon pulse labeling, was calculated from the  $\delta^{13}\text{C}$  values as described in Ruehr et al. (2009), using the background  $\delta^{13}\text{C}$  values measured on the non-labeled experimental units before the pulse labeling (plant  $\delta^{13}\text{C}=-28.0\text{‰}$ , substrate  $\delta^{13}\text{C}=-27.1\text{‰}$ , below-ground-respired  $\text{CO}_2$  measured as  $\text{SrCO}_3$   $\delta^{13}\text{C}=-25.6\text{‰}$ ).

Mycorrhizal growth, P uptake, and rhizosphere respiration responses (MGR, MPR, and MRRR, respectively) for each pot were calculated according to the following

equations, following the concept of Cavagnaro et al. (2003):

$$\text{MGR}_{t,r}[\%] = \frac{\text{DW}_{t,r} - \text{DW}_{\text{NM}}}{\text{DW}_{\text{NM}}} \times 100 \quad (2)$$

$$\text{MPR}_{t,r}[\%] = \frac{\text{PC}_{t,r} - \text{PC}_{\text{NM}}}{\text{PC}_{\text{NM}}} \times 100 \quad (3)$$

$$\text{MRRR}_{t,r}[\%] = \frac{\text{CO2}_{t,r} - \text{CO2}_{\text{NM}}}{\text{CO2}_{\text{NM}}} \times 100 \quad (4)$$

where DW stands for total dry weight of the plants (shoot and roots combined), PC stands for total P content of the plants (shoot and roots combined),  $\text{DW}_{\text{NM}}$  and  $\text{PC}_{\text{NM}}$  represent the means of dry weight and P content of non-mycorrhizal treatment without P supplementation, respectively.  $\text{CO2}$  stands for  $\text{CO}_2$  released from the belowground compartment during a unit of time.  $\text{CO2}_{\text{NM}}$  represents the mean  $\text{CO}_2$  released during a unit of time from the non-mycorrhizal treatment having comparable shoot biomass to the respective mycorrhizal treatment.  $t$  stands for the treatment, and the  $r$  for the replicate.

Furthermore, mycorrhizal rhizosphere respiration response, adjusted for differences in size of root systems between the mycorrhizal and non-mycorrhizal treatments ( $\text{MRRR}_{\text{Adj}}$ ), was calculated as follows:

$$\text{MRRR}_{\text{Adj},t,r} = \left( \frac{\text{CO2}_{t,r} - \text{CO2}_{\text{NM}}}{\text{CO2}_{\text{NM}}} \times 100 \right) \times \frac{\text{DW}_{\text{roots}_{\text{NM}}}}{\text{DW}_{\text{roots}_{t,r}}} \quad (5)$$

where  $\text{DW}_{\text{roots}_{\text{NM}}}$  represents mean root biomass of the non-mycorrhizal treatment having comparable shoot biomass to the respective mycorrhizal treatment, and  $\text{DW}_{\text{roots}_{t,r}}$  stands for root biomass of the individual mycorrhizal plants.

Finally, a coefficient describing mycorrhizal cost-to-benefit ratio ( $\text{CBCoeff}$ ) using the same metric for the costs and the benefits (i.e., carbon allocated underground and increase in net plant C fixation due to mycorrhiza formation, respectively) was calculated as follows:

$$\text{CBCoeff}_{t,r} = \frac{\frac{^{13}\text{C}_{\text{bg}}}{^{13}\text{C}_{\text{tot}}}}{\frac{^{13}\text{C}_{\text{NM-P}}}{^{13}\text{C}_{\text{tot}}}} = \frac{^{13}\text{C}_{\text{bg}} \times ^{13}\text{C}_{\text{NM-P}}}{^{13}\text{C}_{\text{tot}}} \quad (6)$$

where  $^{13}\text{C}_{\text{bg}}$  represents excess  $^{13}\text{C}$  allocated belowground (i.e., sum of the excess  $^{13}\text{C}$  in roots, substrate and in the  $\text{CO}_2$  emitted from the rhizosphere), whereas  $^{13}\text{C}_{\text{tot}}$  stands for excess  $^{13}\text{C}$  in the whole experimental unit (i.e., sum of excess  $^{13}\text{C}$  in shoot and all belowground compartments), and  $^{13}\text{C}_{\text{NM-P}}$  represents the mean excess  $^{13}\text{C}$  found in the non-mycorrhizal treatment without P supplementation.

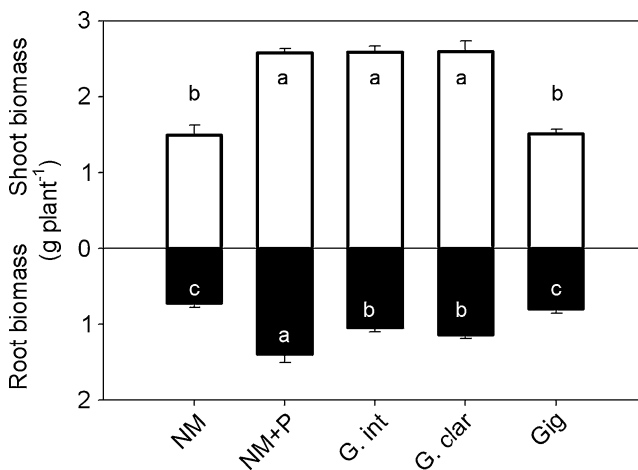
The data were analyzed using Statgraphics Plus for Windows version 3.1. Analysis of variance (ANOVA) was carried out to test differences between treatment means and multiple range tests (least significant difference-based Fischer's test) followed significant ANOVAs.

## Results

### Plant biomass production, root architecture and AM fungal development

According to the biomass accumulation in the plant shoots, two treatment groupings were characterized: The non-mycorrhizal plants without P addition produced similar shoot biomass as the plants inoculated with *G. margarita*, whereas plants inoculated with either of the *Glomus* spp. produced similar shoot biomass as the non-mycorrhizal plants receiving supplemental P (Fig. 2). Therefore, mycorrhizal growth response was high for the two *Glomus* spp., and close to zero for the *Gigaspora*-inoculated plants (Table 2).

Root biomass was higher in the non-mycorrhizal plants supplemented with P than in the *Glomus*-inoculated plants, whereas the root biomass was comparable between the *Gigaspora*-inoculated and the non-mycorrhizal plants without P supplementation (Fig. 2). Root length was reduced in the *Gigaspora*-inoculated plants, whereas it remained unaffected by inoculation with any of the two *Glomus* spp., as compared with the non-mycorrhizal control without



**Fig. 2** Dry biomass of the medic plants 49 days after planting. Bars represent means of five replicates accompanied by standard errors. The treatments are non-mycorrhizal control without P addition (NM), non-mycorrhizal control supplemented with 30 mg P pot<sup>-1</sup> (NM+P), and inoculation with one of the three AM fungal species: *Glomus intraradices* (*G. int*), *Glomus claroideum* (*G. clar*), and *Gigaspora margarita* (*Gig*). Different letters indicate significant differences between treatment means according to least significant difference-based *F* test following significant ANOVA ( $p < 0.05$ )

**Table 2** Plant mycorrhizal responses: growth response (MGR), phosphorus uptake response (MPR), rhizosphere respiration response (MRRR), rhizosphere respiration response adjusted for differences in root biomass (MRRRA<sub>adj</sub>), and the cost-benefit coefficient (CBC<sub>coeff</sub>)

AM fungal species	MGR <sup>a</sup> (%)	MPR <sup>b</sup> (%)	MRRR <sup>c</sup> (%)	MRRRA <sub>adj</sub> <sup>d</sup>	CBC <sub>coeff</sub> <sup>e</sup>
<i>Glomus intraradices</i>	+63.9±5.45 a	+154±7.65 a	+23.6±4.25 b	+31.0±4.78 a	1.18±0.03 b
<i>Glomus claroideum</i>	+68.3±7.93 a	+111±5.43 b	+7.12±2.41 c	+8.58±2.61 b	1.24±0.12 b
<i>Gigaspora margarita</i>	+3.65±5.01 b	+16.2±3.69 c	+37.8±2.96 a	+35.2±3.38 a	1.95±0.04 a
ANOVA <sup>f</sup>	*	*	*	*	*

Values are means±standard errors ( $n=5$ ), different letters within a column indicate significant differences ( $p<0.05$ ) between the means

\* $p<0.001$

<sup>a</sup> Biomass of mycorrhizal plant as compared with the mean of non-mycorrhizal treatment without P addition

<sup>b</sup> P content of mycorrhizal plant as compared with the mean P content of non-mycorrhizal treatment without P addition

<sup>c</sup> Rhizosphere respiration of mycorrhizal plant (mean of hourly values measured on five subsequent days) as compared with the mean rhizosphere respiration of non-mycorrhizal treatment with similar plant biomass. Therefore, *Gigaspora* compares with non-mycorrhizal treatment without P supplementation, whereas both *Glomus* spp. compare with the non-mycorrhizal treatment supplemented with 30 mg P pot<sup>-1</sup>

<sup>d</sup> Corresponds to the MRRR corrected for differences in root biomass between mycorrhizal treatment and the respective non-mycorrhizal control

<sup>e</sup> Ratio of mycorrhizal costs, expressed as proportion of recently assimilated CO<sub>2</sub> (labeled with <sup>13</sup>C) allocated underground, to the benefits expressed as increase in photosynthesis of the plant (measured as <sup>13</sup>CO<sub>2</sub> assimilation by the plants) as compared with the non-mycorrhizal control treatment without P supplementation

<sup>f</sup> Analysis of variance comparing treatment means

P addition (see Electronic supplementary materials, Table S3). On the other hand, average root diameter was higher in the plants inoculated with the two *Glomus* spp. and unaffected by *Gigaspora* inoculation, as compared with the non-mycorrhizal plants without P addition.

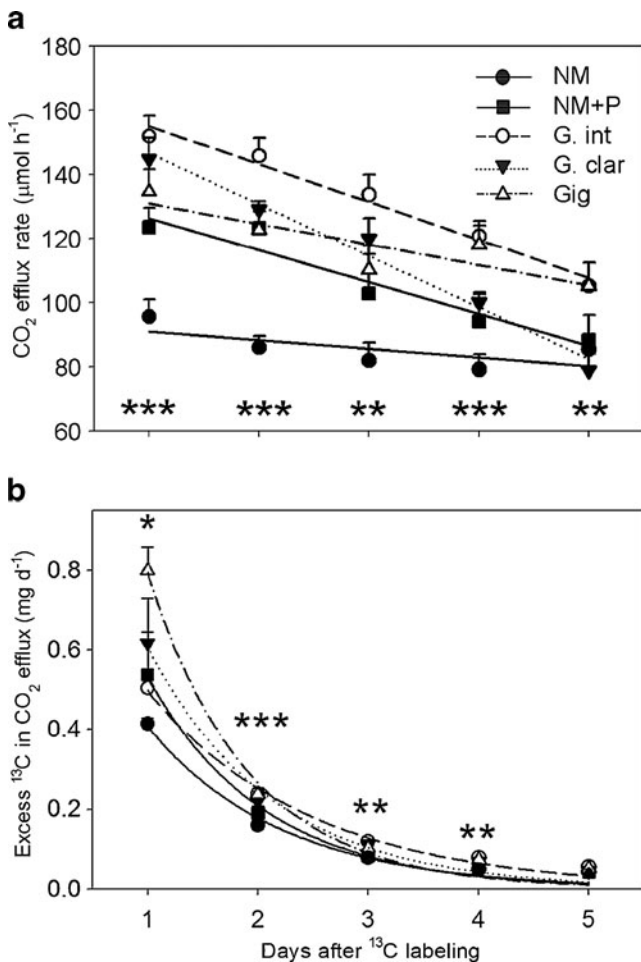
High levels of root colonization by mycorrhizal fungi (32–98% root length colonized) were achieved in the roots of medic inoculated by any of the three AM fungal species, though the extent of root colonization was different between the different AM fungi (see Fig. S2 in the Electronic supplementary materials). Large amounts of hyphae were observed in the rooted substrate of plants inoculated with *Gigaspora*, whereas the highest values of the hyphal length density in the root-free zone were in the *G. intraradices*-inoculated pots (see Fig. S2 in the Electronic supplementary materials). Molecular quantification of mycorrhizal development confirmed no cross-contamination between the different fungal treatments. Root colonization as assessed by the molecular markers was stronger for *G. intraradices* than for the other two fungal species. In contrast, the strength of molecular signal for mycorrhizal biomass in the substrate was similar for all the three AM fungal species (see Table S2 in the Electronic supplementary materials).

Belowground respiration, <sup>13</sup>C allocation and mycorrhizal C costs

Rates of CO<sub>2</sub> release from the belowground compartment were different between treatments (Fig. 3a). CO<sub>2</sub> efflux rates were lowest for the non-mycorrhizal plants without P addition, whereas the greatest values were found in the *G.*

*intraradices*-inoculated pots (Fig. 3a). In all treatments, the amount of CO<sub>2</sub> respired by the rhizosphere per unit of time decreased throughout the monitoring of the CO<sub>2</sub> efflux (Fig. 3a). Mycorrhizal rhizosphere respiration response was greatest for the plants inoculated with *Gigaspora* and lowest for the plants inoculated with *G. claroideum*, whereas intermediate values were observed for the plants inoculated with *G. intraradices* (Table 2). The rhizosphere respiration response adjusted for differences in root biomass between mycorrhizal and the respective non-mycorrhizal treatments reached comparably high values for both *Gigaspora* and *G. intraradices*-inoculated plants, whereas it was significantly lower for *G. claroideum* (Table 2). The cost-benefit coefficient was higher for *Gigaspora* as compared with any of the two *Glomus* spp. treatments (Table 2).

Highest rhizosphere <sup>13</sup>C efflux values were found during the first day after labeling, thereafter the <sup>13</sup>C emission decreased sharply for all treatments (Fig. 3b). A clear tendency towards greater <sup>13</sup>C emissions from the rhizosphere was observed in the mycorrhizal treatments as compared with the non-mycorrhizal plants (Fig. 3b). Particularly, the efflux of <sup>13</sup>C from the rhizosphere of *Gigaspora*-inoculated plants was significantly higher than for any of the non-mycorrhizal treatments during the first, second and fourth day following the labeling, whereas during the third day after labeling, *Gigaspora*-inoculated plants significantly differed only from the non-mycorrhizal treatment without P addition. For the plants inoculated with either *Glomus* spp., <sup>13</sup>C efflux from the rhizosphere was not different from any of the non-mycorrhizal treatments during the first day after labeling. During the second day post-labeling, rhizosphere <sup>13</sup>C efflux



**Fig. 3** Rates of CO<sub>2</sub> efflux from the rhizosphere (a), and excess <sup>13</sup>C in the CO<sub>2</sub> emitted from the rhizosphere (b) during 5-day chase period following the pulse labeling with <sup>13</sup>CO<sub>2</sub>. Mean values of five replicates are shown, accompanied by standard errors. Abbreviations as in Fig. 2. Significances of the analyses of variance for differences amongst treatment means at each separate day are shown: \*0.05 > p ≥ 0.01; \*\*0.01 > p ≥ 0.001; \*\*\*p < 0.001. Linear and exponential regression models were used in (a) and (b), respectively, to indicate temporal changes of the measured variables in each treatment during the 5-day chase period

was higher for *G. intraradices*-inoculated plants as compared with both non-mycorrhizal treatments, whereas *G. claroideum* treatment only differed from the non-mycorrhizal plants without P supplementation. During the third and fourth day post-labeling, the <sup>13</sup>C emissions from the rhizosphere of plants inoculated by any of the *Glomus* spp. were higher than those from any of the non-mycorrhizal treatments.

The sum of <sup>13</sup>C originating from the <sup>13</sup>CO<sub>2</sub> labeling and measured in the different compartments of the experimental unit (i.e., pot with a plant) was proportional to the biomass of the plants growing in the pots. Namely, less <sup>13</sup>C originating from the isotope labeling was detected in the pots where non-mycorrhizal plants without P addition and

the *Gigaspora*-inoculated plants were grown than in the other treatments (Table 3). Within each of these two groups, the mycorrhizal treatments tended to contain more <sup>13</sup>C from the <sup>13</sup>CO<sub>2</sub> labeling than their non-mycorrhizal counterparts (*Gigaspora*, +10%; *G. intraradices*, +7.8%; and *G. claroideum*, +6.6%), though the differences between the mycorrhizal and the respective non-mycorrhizal treatments were not significant at the p < 0.05 levels. A larger fraction of the excess <sup>13</sup>C per experimental unit appeared in the CO<sub>2</sub> released from the rhizosphere of the *Gigaspora*-inoculated plants (27.4%) than in any other treatment (Table 3). In the pots with *Gigaspora*-inoculated plants, a greater portion of the <sup>13</sup>C from labeling was stored in the substrate than in the other treatments, whereas the portion of <sup>13</sup>C remaining in the shoots of *Gigaspora*-colonized plants reached the lowest values among all the treatments (Table 3).

Analysis of the effect of mycorrhizal symbiosis on the excess <sup>13</sup>C respired belowground, calculated in an analogous way as the mycorrhizal respiration costs (Eq. 4), showed that the differences between the three mycorrhizal treatments were only marginally significant (p = 0.064). The rhizosphere of *Gigaspora*-colonized plants emitted about 39% more <sup>13</sup>C than the rhizosphere of the non-mycorrhizal plants without P supplementation and the figures for *G. intraradices* and *G. claroideum* were about +9.6% and +17.3%, respectively (as compared with the non-mycorrhizal plants supplemented with P). If differences in root biomass between mycorrhizal and the respective non-mycorrhizal treatments were taken into account (analogous to Eq. 5), the excess <sup>13</sup>C values were not different between the AM fungal species treatments (p = 0.33), though the trend remained the same as above (*Gigaspora*, +38%; *G. claroideum*, +24%; and *G. intraradices*, +13%).

#### Phosphorus acquisition and mycorrhizal P benefits

Phosphorus content of the *Gigaspora*-inoculated plants was similar to that of the non-mycorrhizal plants without P supplementation (Fig. 4a). Greater amounts of P accumulated in the two *Glomus*-inoculated treatments, whereas non-mycorrhizal plants supplemented with P accumulated the greatest amounts of P (Fig. 4a). Greater amounts of P were found in the shoots of *G. intraradices*-inoculated plants than in those inoculated with *G. claroideum*. The differences in P content within roots of the two *Glomus*-inoculated treatments were not significant from each other (Fig. 4a). Mycorrhizal P uptake response was greater in plants inoculated with *G. intraradices* than in those inoculated with *G. claroideum*. Mycorrhizal P uptake response of the *Gigaspora*-inoculated plants was close to nil (Table 2).

Only the plants inoculated with any of the two *Glomus* spp. acquired significant amounts of P from the root-free



**Table 3** Excess  $^{13}\text{C}$  in the experimental units (i.e., pots with plants) and allocation of the  $^{13}\text{C}$  into the different compartments of the units 5 days after the  $^{13}\text{CO}_2$  labeling (calculated per pot)

Treatment	$^{13}\text{C}$ excess (mg unit $^{-1}$ )	Allocation of the excess $^{13}\text{C}$ (%)				
		Shoot	Roots	Substrate-rooted zone	Substrate-root-free zone	Substrate $\text{CO}_2$ efflux
NM <sup>a</sup>	4.20±0.32 b	66.4±2.22 b	12.6±0.71 a	2.82±0.43 b	0.027±0.003	18.1±1.36 b
NM+P <sup>b</sup>	5.93±0.11 a	73.6±1.18 a	8.94±0.48 b	2.10±0.41 b	0.019±0.003	15.3±1.11 b
<i>Glomus intraradices</i>	6.39±0.10 a	72.0±0.63 a	9.20±0.45 b	3.20±0.75 b	0.029±0.001	15.6±0.54 b
<i>Glomus claroideum</i>	6.32±0.32 a	70.5±2.85 a, b	9.38±0.88 b	2.94±0.64 b	0.022±0.005	17.2±2.38 b
<i>Gigaspora margarita</i>	4.61±0.12 b	53.5±1.03 c	13.4±0.46 a	5.62±0.61 a	0.032±0.003	27.4±1.00 a
ANOVA <sup>c</sup>	***	***	***	**	*	***

Allocation of  $^{13}\text{C}$  into the substrate  $\text{CO}_2$  efflux represents a cumulative value of daily measurements over 5 days following the pulse labeling. Values are means±standard errors ( $n=5$ ), different letters within a column indicate significant differences between the means. Different letters indicate significant differences between treatment means ( $p>0.05$ ) following significant ANOVA

\* $p\geq 0.05$ ; \*\* $0.01>p\geq 0.001$ ; \*\*\* $p<0.001$

<sup>a</sup> Non-mycorrhizal control without P addition

<sup>b</sup> Non-mycorrhizal control supplemented with 30 mg P pot $^{-1}$

<sup>c</sup> Analysis of variance comparing treatment means

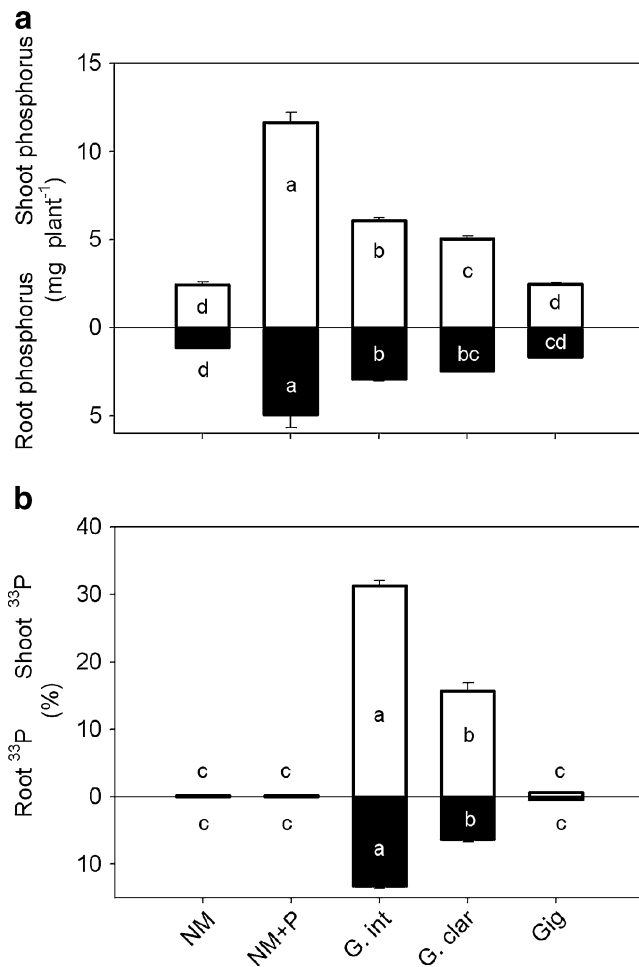
compartment, as revealed by activity of the  $^{33}\text{P}$  isotope in the plants (Fig. 4b). Although the radioactivity in the *Gigaspora*-inoculated plants was rather low and not significantly different from the non-mycorrhizal treatments (Fig. 4b), it was still about one order of magnitude higher than in the non-mycorrhizal treatments. The ratio of radioactivity contained in the roots to that in the shoots was significantly higher for the *Gigaspora*-inoculated plants than for the *Glomus*-inoculated ones (see Table S4 in the Electronic supplementary materials).

## Discussion

We found large differences in P acquisition of the plants colonized by different AM fungal species. *Gigaspora* did not confer any significant P acquisition benefits to the plants, whereas both of the *Glomus* spp. improved plant P acquisition, although not to such a level as observed in the P-supplemented non-mycorrhizal treatment. The observed differences between the two *Glomus* spp. can be explained by the known differences in P acquisition strategies of these AM fungal species (Jansa et al. 2005; Thonar et al. 2011). Namely, the extension of hyphae into a root-free zone differs between the two *Glomus* species, as also illustrated by differential acquisition of the labeled P from the root-free zone in this study. On the other hand, since the root-free zone represented only about 5% of the substrate volume, differences in P acquisition from the root-free substrate cannot fully explain the differences observed in total P acquisition of the plants between the

two *Glomus* treatments. It appears that *G. intraradices* acquired P more efficiently from the substrate than *G. claroideum*, due to either greater mycelium length densities as compared with *G. claroideum*, greater density of P transporters on the mycelium surface, and/or different kinetics of P acquisition (i.e., higher affinity of P transporters). Quite hypothetically, the different AM fungal species may also have associated with different P-solubilizing microbes (Toljander et al. 2006; Jansa et al. 2011), thus gaining access to P pools which would not normally be easily bioavailable.

The observed absence of P acquisition benefits to the plants by *Gigaspora* needs careful examination. Although the mycelium networks of *Gigaspora* were very dense in the root compartment in this study, *Gigaspora* poorly penetrated the meshes, used for construction of the root-free zones. This is consistent with previous studies examining the role of *Scutellospora* and *Gigaspora* in P acquisition by plants (e.g., Smith et al. 2004). This is either because the *Gigaspora* does not extend far enough from the roots or because the (generally thick) hyphae of this species cannot penetrate through the small mesh holes for one reason or another. In either case, low transfer of  $^{33}\text{P}$  from the root-free patch to the plants does not yet exclude existence of mycorrhizal P acquisition pathway in the *Gigaspora*-colonized plants, which would still be operational and efficient if the plant down-regulated its own P transporters in rhizodermis/root hairs and largely relied on P acquisition through the mycorrhizal mycelium (as discussed by Smith et al. 2009). However, careful molecular analyses of gene expression



**Fig. 4** Phosphorus contents of the medic plants (**a**) and percentage of the applied  $^{33}\text{P}$  activity recovered in the plant biomass (**b**) 49 days after planting. Bars represent means of five replicates accompanied by standard errors. Abbreviations as in Fig. 2. Different letters indicate significant differences between treatment means according to least significant difference-based  $F$  test following significant Kruskal–Wallis test ( $p < 0.05$ )

of both plant and fungal P transporters would be required for more understanding of this system. Notably, no P transporter has yet been described for *G. margarita*, whereas several P transporters are known for *M. truncatula* (Liu et al. 2008). Another issue that requires particular attention is the rate of P transfer from the fungus to the plant. It is still possible that P delivery to the plant from *Gigaspora* is efficient, but slower or simply delayed as shown already by Jakobsen et al. (1992b) for *Scutellospora*, further discussed by Boddington and Dodd (1999) for various Gigasporaceae, or more recently demonstrated for the same isolate of *G. margarita* as in this study by Thonar et al. (2011). In the Thonar et al. study, it was shown that *G. margarita* transiently immobilized freshly injected  $^{32}\text{P}$  in its hyphae before it was delivered to the plants. This notion would

also be supported by the fact that the little amounts of  $^{33}\text{P}$  acquired by *Gigaspora* from the root-free zone in this study were effectively immobilized in the roots, most likely in the fungal structures. In consequence, the  $^{33}\text{P}$  was not further distributed between roots and shoots in a similar proportion as for the two *Glomus* spp. Whether this P immobilization is an evolutionary strategy of resource hoarding (Kiers et al. 2010) or whether the *Gigaspora* was metabolically P limited and required the P taken up from the substrate primarily to cover its own needs, remains currently unclear.

The  $\text{CO}_2$  efflux from the rhizosphere of plants colonized by *Gigaspora* was increased by nearly 40% as compared with the non-mycorrhizal plants with the same biomass of shoots and roots and with similar rates of C fixation. Even if the slight, and on average not significant, differences in root biomass of the *Gigaspora*-colonized and non-mycorrhizal plants without P supplementation were corrected for, the mycorrhizal plants still showed some 35% higher rhizosphere respiration rates than their non-mycorrhizal counterparts. In contrast, the correction for root biomass differences between the two *Glomus* spp. treatments and their respective non-mycorrhizal control showed important influence on the results. Without the correction, the increases in rhizosphere  $\text{CO}_2$  efflux due to *Glomus* spp. appeared consistently smaller than the effect of *Gigaspora*. After correcting for differences in the root biomass between the treatments, *G. intraradices*-induced  $\text{CO}_2$  efflux increment appeared as high as that of *Gigaspora*. The effect of *G. claroideum* on rhizosphere  $\text{CO}_2$  efflux was in all cases the lowest among the three AM fungal species tested, indicating that the C demand of this fungus was low as compared with the other AM fungal species.

The gradual decrease in the amounts of  $\text{CO}_2$  respired from the rhizosphere over the several days following the pulse labeling was surprising. This decline was steeper for plants that were larger (i.e., non-mycorrhizal plants supplemented with P and the plants inoculated by any of the *Glomus* spp.) than for the *Gigaspora*-inoculated or non-mycorrhizal plants without additional P. Since we demonstrated that the underground compartments of our experimental units were gas-tight (see [Electronic supplementary materials](#) for details), the reason cannot simply be a diffusion of  $\text{CO}_2$  out of the units. More likely, the rhizosphere suffered from a heat stress as the foil sealing the rhizosphere was transparent and thus creating a chamber where a local glasshouse effect could have developed. Two pieces of evidence would support this notion—elevated temperature inside the rhizosphere compartments were perceived when changing the hydroxide traps, and some of the largest plants developed unspecific weaning symptoms towards the end of the

experiment. Neither of these effects was quantified, however. An alternative explanation is dropping oxygen concentration inside the rhizosphere chamber due to respiration of roots, mycorrhiza and other soil microbes, resulting in (partly) anoxic conditions in the chambers. Unfortunately, the oxygen concentration inside the rhizosphere chambers was not quantified so this remains only a speculation.

Using  $^{13}\text{C}$  pulse-chase labeling allowed several interesting insights into the C dynamics across the different AM fungal species colonizing the medic plants. The patterns of  $^{13}\text{C}$  release from the rhizosphere suggest that the *Gigaspora*-inoculated plants released the freshly fixed carbon somehow faster than the two *Glomus* spp. Our data also confirm that the 5-day chase period was sufficient to collect most of the  $^{13}\text{C}$  appearing in the rhizosphere respiration on a short run, which is consistent with previous evidence (Jakobsen and Rosendahl 1990; Johnson et al. 2002). The amount of  $^{13}\text{C}$  emitted from rhizosphere of any of the treatments during the fifth day after labeling was very low compared with the first day post-labeling, and no differences were observed between the different plant treatments with respect to the rhizosphere  $^{13}\text{C}$  emissions after the fourth day post-labeling, which justifies the length of the chase period for the sake of this study.

Further insights are provided by analyzing the excess  $^{13}\text{C}$  per experimental unit (i.e.,  $^{13}\text{C}$  in plant biomass and all other measured compartments). Admittedly, the  $^{13}\text{C}$  released through shoot respiration over the 5 day  $^{13}\text{C}$ -chase period should be included for these figures to be considered a complete C budget. Nevertheless, here we used the excess  $^{13}\text{C}$  per experimental unit as a proxy for plant photosynthesis (~net photosynthetic productivity). We are aware of possible bias this may have introduced for the interpretation of the results, though there are a number of reasons to believe that this approach is sufficiently justified: (1) All the plants were kept under very standardized environmental conditions. (2) The plants suffered no uncontrolled abiotic or biotic stresses except the assigned treatments. (3) They received all nutrients in excess except P. (4) The shoot (i.e., photosynthetically relevant) biomass of our non-mycorrhizal controls corresponded to the mycorrhizal treatments. Additionally, we did not have the technical option to concurrently measure shoot respiration on so many units.

The comparison of the  $^{13}\text{C}$  excess in mycorrhizal plants with that of the non-mycorrhizal controls of the same shoot biomass, yields numbers that can be regarded as an indicator for the photosynthetic sink stimulation due to mycorrhiza formation—about 10% for *Gigaspora* and between 6% and 8% for the two *Glomus* spp. These are quite realistic values, corresponding well to the records in

literature (Wright et al. 1998; Kaschuk et al. 2009 and references therein). Interestingly, the differences between the mycorrhizal treatments and their relevant controls seem to be overcast by a large variation between individual experimental units and they do not stand as significant when comparing the  $^{13}\text{C}$  excess across treatments. For the plants inoculated by *Gigaspora*, significantly less  $^{13}\text{C}$  was retained in the shoots and a greater fraction of excess  $^{13}\text{C}$  was transported belowground than in the non-mycorrhizal plants. This provides the ultimate proof of a significant C drain belowground due to the presence of this fungus in medic roots. This is consistent with large C expenditure associated with root colonization of cucumber by *S. calospora*, a close relative to *Gigaspora* sp., as demonstrated previously (Pearson and Jakobsen 1993). The C drained belowground appeared mainly in two compartments in our study – in the substrate of the rooted zone and in the respired  $\text{CO}_2$ . The former is most likely due to incorporation of  $^{13}\text{C}$  into the dense hyphal networks around the roots, and the latter is consistent with the rhizosphere respiration measurements and with the observation of rapid  $^{13}\text{C}$  flux through the system upon monitoring the  $^{13}\text{C}$  efflux as  $\text{CO}_2$  from the rhizosphere.

Although the photosynthesis sink strength stimulation was low for *G. claroideum*-colonized plants, the fraction of  $^{13}\text{C}$  in the respired  $\text{CO}_2$  tended to be higher for *G. claroideum* than for *G. intraradices*, whereas the opposite trend was observed for  $^{13}\text{C}$  in the substrate containing extraradical mycelium of the two *Glomus* spp. To reduce the variability of results, it might be relevant in the future to look at the excess  $^{13}\text{C}$  in the pure mycorrhizal structures (hyphae, vesicles, and spores), isolated from both the roots and the substrate, and at the  $\text{CO}_2$  respired by the AM fungal hyphae only. Measurements of  $^{13}\text{C}$  efflux from a root-free compartment are technically possible (Heinemeyer et al. 2006) but will require major technical adaptation, resulting in a lower number of experimental units that could be concurrently monitored. Additionally, such an approach is not suitable for AM fungal species that do not colonize the root-free patches efficiently, e.g., the *Gigaspora*.  $^{13}\text{C}$  allocation into AM fungal biomass could also be assessed by measuring  $^{13}\text{C}$  incorporation into mycorrhizal signature molecules such as specific fatty acids (Olsson and Johnson 2005). This would overcome the need for purification of AM fungal biomass from colonized roots (Saito 1995), which is technically very demanding and provides low yields. Using signature fatty acids may, however, require further calibrations with respect to the choice of the compounds or the concentration of these signatures in mycorrhizal biomass, especially for non-*Glomus* AM fungal species (Jabaji-Hare 1988; Jansa et al. 1999).

In the past, the mycorrhizal costs and benefits were often expressed in different units. For example, P acquisition

benefits to the plants were defined as the increase in plant P content as compared with the non-mycorrhizal control exposed to the same soil and environmental conditions. Mycorrhizal C costs, in contrast, were usually expressed as an increase in the C fraction allocated belowground or the increase in C respired from the rhizosphere. Obviously, for assessment of mycorrhizal C costs it is then important to compare plants with similar characteristics with respect to plant C fixation such as overall biomass production, biomass allocation to roots, similar nutrient status and/or other features. Consequently, the C cost and P benefit calculations require different references (controls). The analyses using these two traditional and separate approaches showed that the P uptake benefits were high for *G. intraradices*, medium for *G. claroideum* and low for *G. margarita*. Mycorrhizal costs as assessed by increase of belowground respiration were high for both *G. margarita* and *G. intraradices* and low for *G. claroideum*. It is important to mention that we were fortunate to have established control treatments which were, in terms of plant biomass production, well comparable with our mycorrhizal treatments. The differences in root biomass between the non-mycorrhizal plants supplemented with P and the two *Glomus* spp., however, still had to be corrected to avoid major interpretation flaws. Similarly, differences in the intensity of root colonization, root architecture and other parameters should have been corrected for a completely unbiased comparison between mycorrhizal and non-mycorrhizal plants to be made.

As a result of using different units for measuring mycorrhizal benefits and costs, the two have been notoriously difficult to compare in the past. Thus, there is an ongoing challenge to develop a protocol for comparing the benefits and costs using the same metric. One of the possibilities is to use a variable which integrates both benefits and costs, such as plant growth rate or biomass production at a certain time point. In our experiment, both *Glomus* species improved biomass production of the experimental plants to a similar extent, which would lead to the conclusion that the cost-to-benefit ratio was similar. And because *G. intraradices* conferred greater P acquisition benefits, it should have also incurred greater C costs than *G. claroideum*—which was indeed in line with the observation. However, given the high measured C costs associated with *Gigaspora* in terms of belowground respiration and the low apparent P uptake benefits to the plants, the absence of a negative growth response of the medic plants to inoculation with *Gigaspora* was surprising and in contrast with other studies, e.g., using wheat as a host plant (Li et al. 2008). To better explain this case, careful examination of the  $^{13}\text{C}$  data was very helpful. Namely, the increase in the excess  $^{13}\text{C}$  appearing in the belowground respiration and in the substrate of *Gigaspora*-containing units nearly equals the 10% increase in C fixation due to

sink strength stimulation as discussed above. This means that the expected growth depression due to the presence of mycorrhizal fungus in its rhizosphere was offset by an increase in photosynthesis by the plant. This is also the most plausible explanation of the observed effects—medic plants associating with *Gigaspora* are receiving hardly any benefits in terms of P uptake, but also are not suffering any growth depression due to C drain belowground. Yet, how such an association can work on evolutionary scales remains unclear. Possibly, the P delivery to the plants at a later time point from the *Gigaspora* mycelium, where it has been transiently immobilized, may in some situations (e.g., competitive pressure, environmental fluctuations) offset the transient lack of benefits. After all, the rapid growth and large biomass production of plants are without doubt very important in agriculture, but are not always strongly associated with plant fitness in natural ecosystems (Grime 2001).

Our results thus demonstrate that sink strength stimulation has, at least in some situations and/or symbiotic partner combinations, a large impact on the outcome of the association of plants with mycorrhizal fungi. As such, it should be taken into consideration as an important parameter describing the functional diversity of arbuscular mycorrhizas. In this respect, it would be very useful to develop a standardized indicator of costs-to-benefits, using the same currency, namely the carbon. The symbiotic benefits would then be expressed as increased photosynthesis of the plant (C fixation) and the costs would be the C supply to the AM fungal structures, or (operationally) increased C drain belowground, as used here for the generation of the so-called cost-to-benefit coefficient. The theoretical outline of such an approach was proposed decades ago (Koide and Elliot 1989), but because of technical difficulties and a lack of well-established and accessible stable-isotope technology to track C fluxes in plant–soil systems, it has not, thus far, been broadly established in the mycorrhizologists community. The experimental system outlined here offers an option for allowing the comparison of many experimental units running concurrently, with moderate experimental costs and without producing long-living ( $^{14}\text{C}$ ) radioactive waste. Few experimental shortcomings encountered in this study should be addressed for the future experimental setup and are briefly outlined below.

Overheating of hermetically sealed rhizosphere compartments will need to be prevented, e.g., by using light-reflective foils or active temperature control of the underground compartments. To offer a better coverage of the rhizosphere respiration measurements, sampling interval should be shorter, e.g., 8 or 12 h (instead of 1 day). More frequent changing of the hydroxide  $\text{CO}_2$  traps would also lead to more frequent exchange of the headspace atmosphere in the rhizosphere compartments. This would better prevent the development of



anoxic conditions in the underground compartment, which may have been a major limitation of the experimental system in this study. The reasons for the high variation between experimental units for some results (especially the excess  $^{13}\text{C}$ ) should be investigated in the future. Alternatively, the effect of high variability between individual plants could probably be minimized by increasing the number of replicates per treatment. This would allow for better quantification of small differences between large (and variable) numbers needed to assess the sink strength stimulation in this study. The establishment of whole plant C budget will also require measuring shoot respiration, in addition to the measurements presented here. This will allow precise assessment of photosynthesis stimulation due to increased sink strength. Consequently, it will allow calculation of true net C costs, i.e., gross costs minus photosynthesis stimulation due to sink strength increase. However, this presents other technical challenges which may impose a limit upon the number of plants which could be observed simultaneously. On the other hand, it should also be possible to assess P uptake into both the plant (net P benefits) and the extraradical mycorrhizal hyphae to obtain the gross P benefits and compare these to the gross C costs as measured in this study. In this respect, it is probably important to mention that there is no proof that all the P measured in the root biomass was in the plant cells as the roots were generally well colonized by the fungi and some P was possibly still in the fungal biomass (this appears particularly the case for *Gigaspora*, see also Thonar et al. 2011), so the values for P benefits as shown in this study are somewhere between the net (P in the plants only) and gross P benefits (P in the plants+AM fungus). We admit that rigorous comparison of costs and benefits should deal with these issues to allow even better insights into mycorrhizal economy.

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