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ORIGINAL PAPER

High functional diversity within species of arbuscular mycorrhizal fungi is associated with differences in phosphate and nitrogen uptake and fungal phosphate metabolism

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

Keywords Arbuscular mycorrhizal symbiosis · Fungal diversity · Glomeromycota · Nitrogen · Nutrient uptake and transport · Phosphate

Introduction

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

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has been demonstrated that both host and fungus can discriminate among their partners, reciprocally rewarding those partners that provide more mutualistic benefit (Kiers et al. 2011).

While the symbiosis is generally positive for the host, mycorrhizal growth responses (MGR) can range from highly beneficial to detrimental (Johnson and Graham 2013; Johnson et al. 1997; Smith and Smith 2013) depending on abiotic factors such as nutrient level (Smith and Smith 2013; Peng et al. 1993; Nouri et al. 2014), and biotic factors such as the identity of the fungal symbiont colonizing the host (Smith et al. 2004). There is a high functional diversity in nutritional benefit, not only among different fungal morphospecies but also among isolates within one morphospecies, and it has been shown that even the genetic diversity in one initial spore can be sufficient for the development of phenotypically different variants of one fungus (Ehinger et al. 2012). While fungal isolates differ greatly in the efficiency with which they provide nutritional benefits to plant hosts (Avio et al. 2006, 2009; Hart and Reader 2002b), there is still a lack in understanding why particular AM fungal isolates are much more beneficial than others.

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

Inorganic N sources taken up by the fungus from the soil are assimilated in the hyphae of the ERM and converted mainly into the basic amino acid arginine (Cruz et al. 2007; Jin et al. 2005). It has been suggested that arginine could bind to the negatively charged poly-P and could be transferred with poly-P from the ERM to the IRM (Cruz et al. 2007; Fellbaum et al. 2012a). In the IRM, poly-P are remobilized and P_i and arginine are released, and the catabolic arm of the urea cycle reconverts arginine back into NH_4^+ (Govindarajulu et al. 2005; Fellbaum et al. 2012a; Tian et al. 2010). P_i and NH_4^+ are then transferred into the mycorrhizal interface and are taken up from the interface by mycorrhiza-inducible plant P and ammonium transporters that are localized in the periarbuscular membrane (Gomez et al. 2009; Guether et al. 2009; Javot et al. 2007; Pumplin et al. 2012).

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

Material and methods

Fungal and plant culture

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

The alfalfa seeds were surface sterilized for 1 min in 7 % bleach and rinsed three times with sterile water, before

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

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

sowing. Plants were grown in pots filled at the bottom with 50 ml autoclaved (twice at 121 °C for 20 min) and pressed Sunshine mix #2 (Sun Gro Horticulture, Vancouver, BC, Canada), which was overlayed with 100 ml of an autoclaved (see above) mixture (1:3:1; *v*:*v*:*v*) of field soil, Turface (Turface Athletics MVP, Profile Products LLC, Buffalo Grove, IL, USA) and washed horticultural sand (Hillview, Nu-Gro IP Inc., Brantford, ON, Canada), 20 ml of non-mycorrhizal or mycorrhizal inoculum (see below), and on top with another 50 ml of the substrate mixture. The field soil was collected at the Long-Term Mycorrhizal Research Site located at the University of Guelph (Canada) (Klironomos 2000), passed through a 5-mm sieve, and air-dried at room temperature. The chemical properties of the field soil (analyzed by the University of Guelph Laboratory Services, ON, Canada) were as follows: 140 mM kg^{-1} total N (measured by LECO FP 428 N analyzer), 0.065 mM kg^{-1} available P (Olsen method), and pH 7.7 (saturated paste method).

The mycorrhizal inoculum for the experiment was produced by growing each fungal isolate with *Sorghum vulgare* (Pers.) var. *sudanense* as host species in pot cultures in a greenhouse at the University of Guelph (Canada). The substrates of these cultures were collected after 5 months, air-dried, and controlled for the presence of viable AM fungal spores of the correct morphotype. To each of the mycorrhizal treatments, 20 ml of inoculum containing AM fungal spores, hyphae and mycorrhizal roots were added. To the non-mycorrhizal controls, 20 ml of substrate and roots of non-mycorrhizal *S. vulgare* cultures or 20 ml of autoclaved fungal inoculum was added. No signs of AM fungal colonization (no

root colonization, no fungal spores) were found in either control treatment, and since both control treatments did not differ statistically in any of the traits studied, they were subsequently pooled into one non-mycorrhizal control group. To minimize differences in the non-AM microbial communities, 1 ml of a microbial wash solution was added to each container. This microbial wash solution was obtained by suspending 20-ml subsamples of each of the AM fungal inocula from the *S. vulgare* cultures (see above) in 2 l of sterile water and by filtering the solution through a 20-μm sieve. All containers were covered with a thin layer of sterile washed sand and arranged in a completely randomized block design in the greenhouse.

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

Analysis of fungal and plant growth characteristics

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

$$\text{MGR in \%} = 100 \times (\text{d.wt. AM plant} - \text{d.wt. mean of controls}) / \text{d.wt. mean of controls}$$

Phosphate and nitrogen analysis

Root and shoot samples were individually homogenized in a tissue grinder (Precellys 24, Cayman Chemical Company, Ann Arbor, USA), and an aliquot of each sample was dried and analyzed for P or N content. For the P analysis, the sample was extracted with 2 N HCl at 95 °C for 1 h (Ohtomo et al. 2004). Additionally, the allocation of P into different P pools in non-mycorrhizal and mycorrhizal root samples was measured following the protocol described by Aitchison and Butt (1973). The samples were dried at 70 °C, weighed, and analyzed for the following P pools: P_i and acid soluble or short-chain poly-P (chain length ≤20 P_i residues) after extraction with ice-cold 10 % TCA, phospholipids after extraction with 100 % ethanol and ethanol/ether (3:1, *v*:*v*), acid-insoluble poly-P (chain length >20 P_i residues) after extraction with 1 M KOH, and DNA-, RNA- and protein-phosphates as residue after extraction of all other pools. The pH of the supernatants of the TCA or KOH extractions containing the acid soluble or acid insoluble poly-P were first neutralized by adding 3 M KOH or 3 M HCl, respectively, and then adjusted to a pH of 4.5 by adding 3 M acetate buffer. The poly-P were then precipitated twice by adding a saturated $BaCl_2$ solution at 4 °C overnight. An aliquot of the poly-P or DNA-, RNA-, protein-phosphate precipitates was diluted in 2 N HCl and heated up to 95 °C for 1 h before analysis. The P content was measured spectrophotometrically at 436 nm after adding ammonium-molybdate-vanadate solution (Ricca Chemical, Arlington, TX, USA) to an aliquot of the sample. The total N content in 3 mg aliquots of the shoots was analyzed by using an isotope mass spectrometer (Sercon, Europa-Scientific, Crewe, UK).

Statistical analysis

The data are based on four biological replicates per AM fungal isolate and eight non-mycorrhizal control plants. Since the results demonstrated high intraspecific variability and the species affiliation for several of the fungal species is uncertain at this point, all fungal isolates were treated as independent variables in all statistical tests. Unless mentioned otherwise, treatment effects are only discussed when they were statistically significant according to one-way ANOVA with isolate as a fixed factor followed by Fisher's least significant difference (LSD) test ($p \leq 0.05$) (biomass data). An ANCOVA was used to confirm the results of the ANOVA analysis and to account for the effects of the continuous covariate (biomass) on the statistical evaluation of the nutritional benefits. The results of these tests are given in the Tables S1 to S7 (see supplementary information).

The fungal isolates were grouped according to their effect on the plant MGR as high, medium, and low performance isolates (see results). When ANOVA and LSD test of the individual fungal isolates demonstrated significant differences between fungal isolates that were related to their symbiotic performance, an additional one-way ANOVA and LSD test was conducted with the fungal isolates grouped according to their performance Correlations among traits were examined by calculating the

Pearson correlation coefficient or a linear regression analysis ($p \leq 0.05$). These results are shown in the Tables S8 to S11. The analytical software UNISTAT 6 (Unistat Ltd., London, U.K.) was used for all analyses.

Results

Effect of different AM fungi on plant biomass

Total biomass of mycorrhizal *M. sativa* plants was higher than that of non-mycorrhizal control plants, but there was a high variability in the mycorrhizal growth response (MGR) across AM fungal isolates (Fig. 1, Table S1). The % increase in total plant biomass ranged from 7.3±10.8 (mean±S.E.M.) in plants colonized with *R. irregulare* QB000 (not significantly higher than the controls) to 207.4±36.4 in plants colonized with *Acaulospora colombiana* NB104C. The intraspecific variability in the MGR between the different isolates of one AM fungal morphospecies was similarly high. For example, two other strains of *A. colombiana* (CL 356 and GA101) did not lead to a significant growth response relative to the non-mycorrhizal controls. Of the fungal isolates tested, the three *Acaulospora morrowiae* isolates led on average to the highest increase (113.8±32.4 %) and the four *Rhizophagus* isolates to the lowest increase in total plant biomass (20.2±15.9 %) (Table S1).

Among the different fungal isolates that were tested, six stood out because they showed several unique characteristics (e.g., in their effect on P and N nutrition) and resulted in the highest increase in total plant biomass relative to all other isolates (i.e., a MGR of more than 170 % relative to the control and more than 65 % higher than the next highest performing isolate with an increase in host biomass of 104 %) (Fig. 1, Tables S1 and S8(1–7)). This group, later referred to as "high performance isolates," included isolates from six different fungal morphospecies, *A. colombiana* NB104C, *Funneliformis mosseae* NB114, *A. morrowiae* FL219B, *Paraglomus occultum* OR924, *Acaulospora scrobiculata* VA104, and *Claroideoglomus etunicatum* MG106. Six isolates led only to small increases in total plant biomass (≤18 %) and did not differ significantly in many characteristics from the non-mycorrhizal controls but differed from the high performance isolates. These "low performance isolates" included *R. irregulare* QB000, *P. occultum* CR102, *Claroideoglomus claroideum* UT159A, *A. scrobiculata* CU

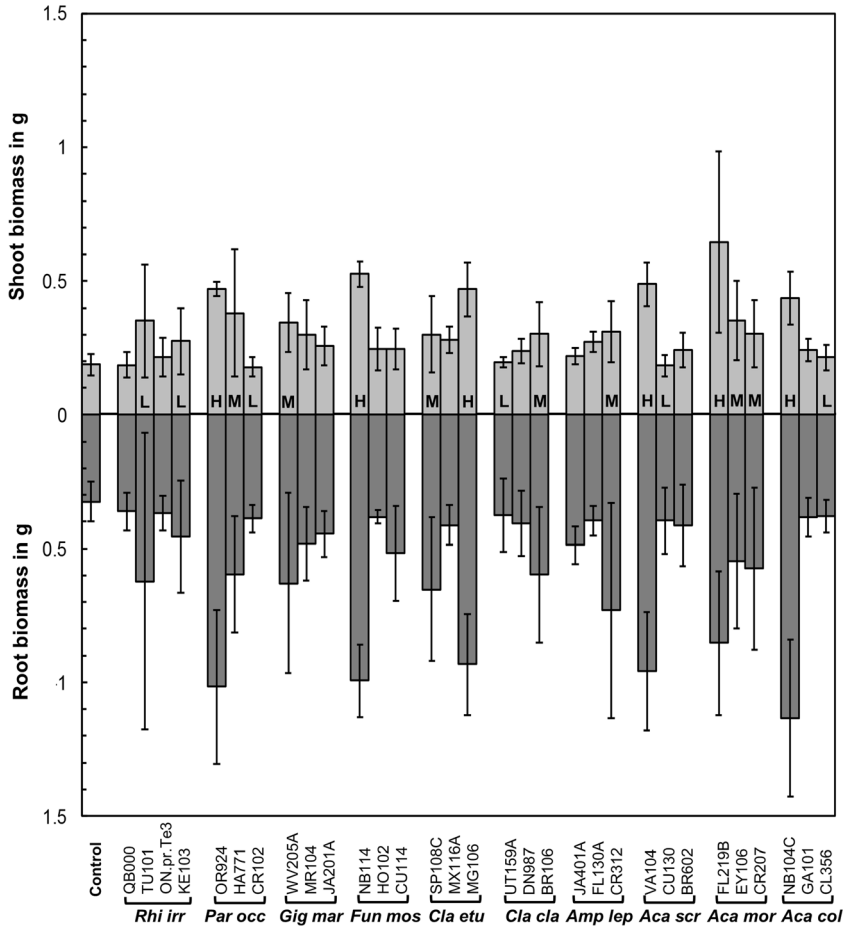

Fig. 1 Plant biomass characteristics of non-mycorrhizal and mycorrhizal *Medicago sativa* plants. The bars show the means ($n=4$) of the dry biomass in gram of roots (*bottom*, *dark grey*) and shoots (*top*, *light grey*) and their respective confidence intervals ($p \leq 0.05$). The *letters in the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

130, *R. intraradices* ON.pr.Te3, and *A. colombiana* CL356 (Table S1). In between the low and high performance isolates, a group of isolates could be identified that significantly increased plant biomass compared to the controls but led to a significantly lower biomass response than the high performance isolates (Fig. 1, Table S1). These "medium performance" isolates led to MGR between 71.7 and 104.0 % and included *Ambispora leptoticha* CR312, *P. occultum* HA771, *Gigaspora margarita* WV205A, *C. etunicatum* SP108C, *A. morrowiae* EY106, *C. claroideum* BR106, and *A. morrowiae* CR207. A high within-treatment variability in plant growth responses was observed for the remaining isolates. Plants colonized by these isolates did not differ significantly from the non-mycorrhizal controls but had a consistently lower biomass response than the high performance isolates.

Correlation between host biomass and P benefits of the AM symbiosis

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

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

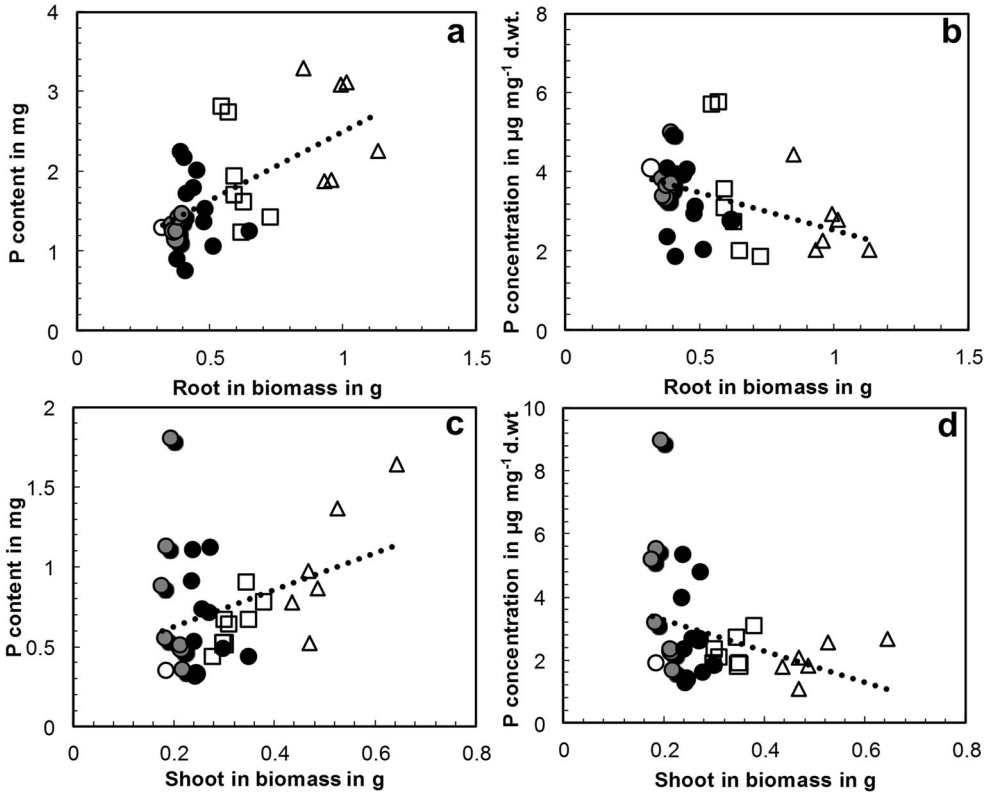

Fig. 2 Correlation between **a**, **b** root or **c**, **d** shoot biomass and P **a**, **c** content or **b**, **d** concentration. Data of the non-mycorrhizal controls are shown as *open circles*, of plants inoculated with high performance isolates as *open triangles*, medium performance isolates as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

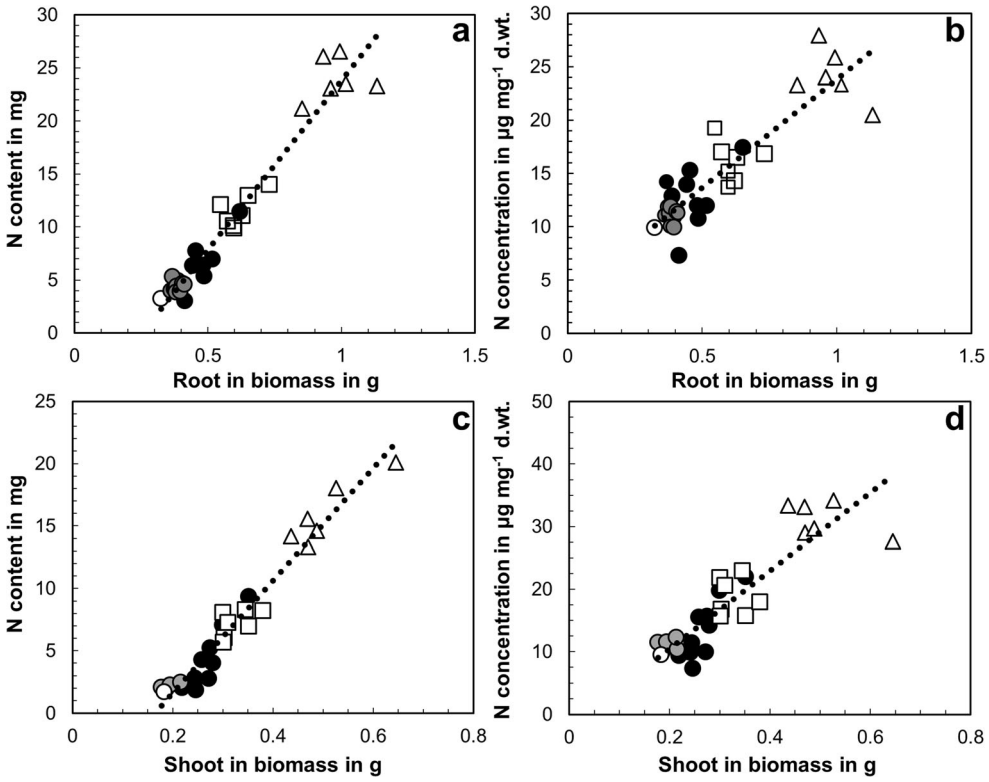

Fig. 3 Correlation between **a**, **b** root or **c**, **d** shoot biomass and N **a**, **c** content or **b**, **d** concentration. Data for the non-mycorrhizal controls are shown as *open circles*, of plants inoculated with high performance isolates as *open triangles*, medium performance isolates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

Correlation between host biomass and N benefits of the AM symbiosis

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

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

Allocation of P in different P pools of the root

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

The metabolically active P_i pool (in %) in the roots was generally lower when plants were colonized with the high performance isolates (except *A. morrowiae* FL219B and *A. scrobiculata* VA104) than in plants that were colonized with the low performance isolates (Fig. S5a, Table S11(3–

6)). The effects of the low performance isolates also differed significantly from that of medium performance isolates, when the isolates were grouped according to their performance, but not when individual isolates were compared (Table S11(5)). Root biomass was negatively correlated with the P_i tissue concentration in the root (Fig. 4a, Table S11(7)) and the P_i tissue concentrations in roots that were colonized with the high performance isolates, and several of the medium performance isolates, were generally lower than in roots that were colonized with the low performance isolates (Fig. S7a, Tables S6 and S11(9–10)). The P_i content in the roots that were colonized with the high performance isolates, however, did not differ significantly from the non-mycorrhizal controls, or those colonized with the low or medium performance isolates (Fig. S6a).

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

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

Discussion

Approximately 200 different AM fungal morphospecies have been described so far, but the genetic and functional diversity among AM fungal strains is much larger than the small species number suggests (Koch et al. 2006; Ehinger et al. 2012). While it is appreciated that colonization by different isolates can lead to different host growth responses (Koch et al. 2006; Ehinger et al. 2012; Munkvold et al. 2004), it is unknown

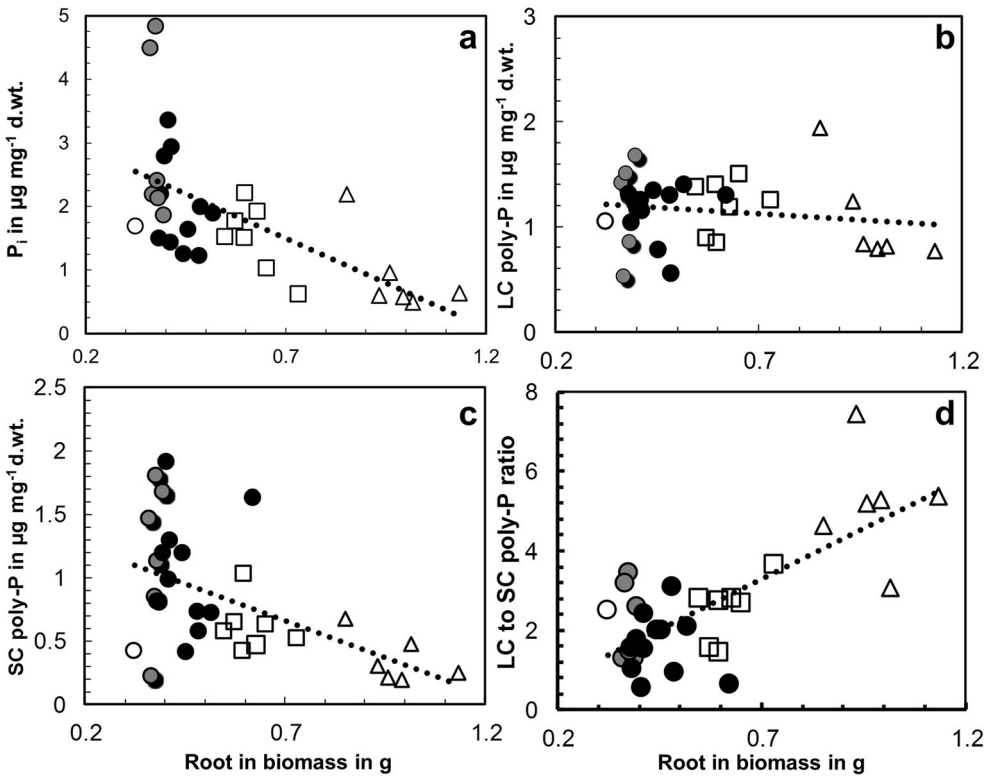

Fig. 4 Correlation between root biomass and tissue concentrations of **a** P_i, **b** long-chain poly-P, **c** short-chain poly-P, and **d** the ratio between long-chain and short-chain poly-P. Data of the non-mycorrhizal controls are shown as open circles, of plants inoculated with high performance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

what causes this high within species functional diversity. Here, the growth response of *M. sativa* was examined after colonization with 31 different fungal isolates from 10 morphospecies to evaluate whether the poly-P metabolism in AM fungi is phylogenetically controlled and whether differences in the efficiency with which AM fungi contribute to nutrient uptake and biomass development can be related to differences in P metabolism.

Based on the high variability in effects on the MGR among AM fungal isolates, the isolates were grouped into three performance levels. High performance isolates led in *M. sativa* to MGR of more than 170 %, medium performance isolates to MGR between 71 and 104 %, and low performance isolates did not lead to significant increases in plant biomass compared to the non-mycorrhizal controls (MGR≤18 %). Fungal isolates within one performance level generally shared several important characteristics (e.g., their effect on P or N nutrition) under the present experimental conditions, and the performance levels were used to better describe these characteristics. However, MGR (or the performance level of an AM fungus) depends on the compatibility between the AM fungal symbiont and its host (Smith et al. 2004) and is strongly context-dependent (Peng et al. 1993). For example, the high performance isolates that were tested here led in *Achillea millefolium* L., and *Bromus inermis* Leyss to relatively low MGR, and in these plant species the intraspecific variability among the different fungal isolates was much less pronounced than in *M. sativa* (Koch et al., unpublished).

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

However, MGR and high P and N levels of *M. sativa* were not related to any of the fungal growth and colonization patterns (Table S2, Koch et al., unpublished). Fungal growth traits have been shown to be evolutionary conserved (Powell et al. 2009), but the present results demonstrate that the effects of AM fungal isolates on host plant growth and P and N uptake are not conserved. This confirms the results of Munkvold et al. (2004) who found that the length-specific hyphal P uptake is rather constant within one fungal species but that the within species variability in hyphal length, as well as effects on shoot growth response and shoot P content, are greater than the between species variability and that these functional characteristics are not aligned with the fungal phylogeny. This asymmetry indicates that the greater effect of some AM fungal isolates on plant P and N nutrition was more likely the result of more efficient P and N uptake systems and/or higher nutrient transport rates to the host. This is consistent with other studies in which no correlation between the dimensions of the ERM and P uptake and/or MGR was found (Hart and Reader 2002a; Smith et al. 2000). A meta-analysis recently revealed that the mycorrhizal colonization is only in part responsible for the high diversity in MGR that can be observed but that AM fungal taxa also differ in their mycorrhizal benefit per unit root length colonized (Treseder 2013). In contrast, in other reports, the functional diversity of AM fungal isolates was related to the dimensions or the interconnectedness of the ERM or to the absolute root length colonized (Avio et al. 2006; Munkvold et al. 2004). Similar to the results of Hart and Reader (2002a), who reported greater host benefits conferred by AM fungal families with larger internal mycelia, there was only a positive correlation between the total biomass of *M. sativa* and an estimate of the total arbuscular volume in the roots.

Several of the AM fungal isolates did not lead to significant biomass or nutritional gains in *M. sativa* compared to the non-mycorrhizal controls (neutral MGR). Neutral MGR have been observed under both non-limiting and growth-limiting levels of P in the soil (Smith and Smith 2013; Peng et al. 1993). However, recent work suggests that AM fungi can also contribute to the P uptake of their host in the absence of positive MGR (Li et al. 2006; Smith et al. 2003). It has been suggested that negative or neutral MGR can be the result of a mycorrhiza-induced suppression of the plant P uptake pathway (via root hairs and epidermis) that is not compensated for by increases in the P uptake via the mycorrhizal uptake pathway (via the ERM and the mycorrhizal interface) (Smith et al. 2011; Smith and Smith 2011). There is evidence that AM fungi differ in their ability to inhibit the plant P uptake pathway. *R. intraradices*, for example, has been shown to nearly completely suppress the plant uptake pathway for P in several plant species, including *Medicago truncatula* (Smith et al. 2004; Grunwald et al. 2009). Of all the AM fungal species tested here, the four *Rhizophagus* isolates led to the lowest MGR (average of 20.2±9.3 %) and the plants did not differ in their biomass from the non-mycorrhizal controls. However, the fact that the P tissue concentration in the shoot and the P_i level in the roots of plants that were colonized with *Rhizophagus*, and some of the other low performance isolates, were significantly higher than in the controls or plants that were colonized with several of the high performance isolates, could indicate that these fungi contributed to the P nutrition of the plants, despite their overall neutral MGR.

The high performance isolates significantly increased the P nutrition of *M. sativa* compared to the non-mycorrhizal controls and the low performance isolates. However, what really set these isolates apart from the non-mycorrhizal controls, and the low and medium performance isolates, was their positive impact on N nutrition. The N tissue concentration in the shoots of the plants that were colonized with the high performance isolates were on average 2.4 times higher and the N content 3.8 times higher than in the non-mycorrhizal controls. While the positive effect of the AM symbiosis on P nutrition has been long known (Smith et al. 2011; Smith and Read 2008), the role that AM fungi play in the N nutrition of their host is still under debate (for review see Smith and Smith 2011). It has been suggested that an improved N status of mycorrhizal plants may simply be a consequence of an improved P nutrition (Reynolds et al. 2005). The present results, however, suggest that the increase in the N nutrition of *M. sativa* by the high performance isolates was not only the result of an improved P nutrition, because both medium and high performance isolates increased the biomass of the plants and increased the P root contents compared to the controls. However, only the high performance isolates increased the N content of the plants and induced a greater biomass response than the medium performance isolates. These results confirm several other studies reporting a substantial contribution of AM fungi to the N nutrition of their host (Toussaint et al. 2004; Tanaka and Yano 2005; Ngwene et al. 2013; Nouri et al. 2014).

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

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

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

Poly-P play an important role for the P but also N transfer from the ERM to the IRM (Cruz et al. 2007; Bücking and Shachar-Hill 2005; Ryan et al. 2007; Viereck et al. 2004). Consistently, the fungal isolates that contributed to both P and N nutrition of *M. sativa* showed the same characteristics in their P metabolism. It is generally hypothesized that long-chain poly-P are first broken down to short-chain poly-P and, subsequently, remobilized by an exopolyphosphatase into P_i that can be transferred across the mycorrhizal interface (Ohtomo and Saito 2005). It has been suggested that long-chain poly-P better represent the long-term storage capacity of P in AM fungal hyphae, whereas short-chain poly-P is a good indicator of P transport to the host (Kiers et al. 2011; Takanishi et al. 2009). The present results seem to be contradictory to this view, because *M. sativa* roots colonized with high performance isolates had reduced levels of short-chain poly-P and a high long-chain to short-chain poly-P ratio. This could indicate that medium and high performance isolates

differ from low performance isolates in their capability to remobilize short-chain poly-P into P_i, but not in their capability to store P in form of long-chain poly-P. The particularly high long-chain to short-chain poly-P ratio in high performance isolates, however, also supports the view that medium and high performance isolates did not differ in their effect on P but in their effect on N nutrition. The high biomass of plants that were colonized with the high performance isolates would first cause a dilution effect of the poly-P pool that is more readily available for the host, which supports the hypothesis that the short-chain poly-P pool is a good indicator for the P transport efficiency to the host (Takanishi et al. 2009).

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

It has recently been shown that even the genetic diversity in one spore can lead to genetically different variants, variable phenotypes, and differences in MGR (Angelard et al. 2010; Ehinger et al. 2012). AM fungal growth traits have been shown to be phylogenetically conserved across the phylum Glomeromycota (Powell et al. 2009) but based on the current classification of the morphospecies that were used in this study, fungal effects on P or N nutrition were not phylogenetically conserved. In contrast, the present results demonstrate that the capability to contribute substantially to host plant benefit is widely spread across the phylum Glomeromycota. The asymmetry in conservatism between AM fungal traits and host plant performance suggests that the fungal adaptability to the host plant also plays an important role in the symbiotic performance of both partners (Smith et al. 2004). This is also supported by the observation that the high performance strains did not consistently show the same symbiotic performance in other host plant species as in *M. sativa* (Koch et al., unpublished).

In conclusion, mycorrhizal benefits are often discussed only in terms of an improved P nutrition and their respective carbon costs, but the results here show that the plant growth response promoted by high performance isolates was related to their positive impact not only on P but also on N nutrition and that the MGR was the result of the sum of these nutritional benefits (P and N) for the plant (Nouri et al. 2014). It has been shown that P in combination with N limitation induces changes in the plant transcriptome that stimulate the AM colonization of plants under P and N stress despite an overall higher P status in mycorrhizal plants (Bonneau et al. 2013). However, in addition to a high efficiency with which P and N are taken up, mycorrhizal growth benefits also depend on the rate with which fungal poly-P are remobilized and nutrients are released into the mycorrhizal interface. The high performance isolates examined here were particularly characterized by a high efficiency with which they took up P and N from the soil, but also by their capability with which they remobilized poly-P and released P and N in the IRM, and transferred these nutrients to their host. Considering the key role that the P metabolism of the fungus plays for P and N transport in the symbiosis, it is crucial to better understand the physiological and regulatory mechanisms that contribute to the high functional diversity in P and N nutrition between the different AM fungal isolates. The results shown here only represent a snapshot of the P allocation into different P pools after 20 weeks of growth. Further experiments with P isotopes in time course experiments in multi-compartment systems are now necessary to track the P uptake by high and low performing isolates and to follow the transport to the plant through the different P pools.

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