

# Arbuscular mycorrhizal fungi pre-inoculant identity determines community composition in roots

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## ABSTRACT

Pre-inoculation of seedlings with commercial, typically non-indigenous, AMF inoculants is common practice in horticultural and land reclamation industries. How these practices influence AMF community composition in pre-inoculated seedlings after they are planted in soil containing a resident AMF community is almost completely unknown. However, there may be important implications regarding success of horticultural practices, as well as unexpected ecological consequences. In this study we exposed *Leucanthemum vulgare* seedlings to five different AMF treatments (pre-inoculation with a representative of *Glomus* group A and *Glomus* group B, one of two *Gigaspora* spp., or no AMF) prior to exposure to a whole-soil, mixed-AMF community inoculum. After a growth period of 75 additional for 28 days, AMF community composition within the roots was analyzed using an approach combining LSU rDNA sequencing and T-RFLP analysis. Our results indicate that the AMF communities that assemble within roots were strongly influenced by AMF pre-inoculant identity. Pre-inoculation with either *Glomus* spp., unlike what was found for *Gigaspora*, greatly restricted numbers of other AMF ribotypes able to subsequently colonize roots after exposure to our Glomeraceae-dominated field soil; this suggested that phylogenetic relatedness and life history strategies may play a role in AMF community assembly. Our results further revealed concurrent changes in AMF community functions, as indicated by differences in plant biomass and foliar nutrients. These results serve to highlight the importance of considering life history differences when designing AMF inoculants and may have important implications regarding the introduction of non-indigenous AMF.

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## 1. Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs that form symbiotic associations with approximately two-thirds of all terrestrial plants. Representing a key interface between plant hosts and soil mineral nutrients, AMF are also known to confer upon their hosts increased resistance to pathogens and other environmental stresses, as well as improved water relations (Smith and Read, 2008).

Due to their salient roles in plant nutrient acquisition, there is considerable interest in using AMF as “bio-fertilizers”. Use of commercial, typically non-indigenous, AMF inoculants is widespread in horticultural and land reclamation industries. This commonly involves growing plants in the presence of one or more AMF species prior to planting in the field with the goal of guaranteeing high abundance and functionality of the AMF community

available to form mutualistic relationships with the plant. Although introduction of non-indigenous AMF species in this manner is sometimes considered ecologically benign (Azcon-Aguilar and Barea, 1997), partially because of expected positive effects on plant establishment or compared to other management practices (e.g. fertilization), very little information is available regarding the potential ecological implications of these practices on which to base management decisions (Schwartz et al., 2006).

In natural ecosystems AMF are typically found as mixed communities with multiple species colonizing any given plant root. Since the functionality of the symbiosis is highly variable and dependent upon the identity of the AMF and host species involved (Johnson et al., 1997; Klironomos, 2003), the composition of AMF species colonizing a given plant has important implications for its fitness. Taken to the level of the plant community, AMF species composition may be a salient determinant of plant community composition (e.g. van der Heijden et al., 1998). Thus, alteration of AMF community composition could have important implications for ecosystem function (Rillig, 2004).

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Although the mechanisms structuring AMF communities are poorly understood, a few studies suggest that interactions between AMF species are important (Abbott and Robson, 1984; Hepper et al., 1988; Pinior et al., 1999; Vierheilig et al., 2000; Scervino et al., 2005; Alkan et al., 2006). Moreover, a recent study (Maherali and Klironomos, 2007) indicates that the phylogenetic relatedness of members of model AMF communities can influence community assembly in plant roots. These results further suggest that colonization of roots by specific AMF species may influence subsequent colonization of other, closely related species.

From a horticultural standpoint, our lack of understanding about how AMF pre-inoculants interact with indigenous AMF communities makes it difficult to disentangle mechanisms behind observed plant growth responses; are they due to direct effects of the pre-inoculated fungus, or are they due to indirect effects, such as alteration of the resident AMF community composition?

This study was designed to test the hypothesis that pre-inoculation of seedlings with different AMF species alters the richness, diversity and functionality of AMF communities that subsequently assemble within roots when exposed to a resident soil community of AMF.

## 2. Materials and methods

### 2.1. Plant and fungal materials

*Leucanthemum vulgare* Lam. (Asteraceae; common name, Ox-eye daisy) was used as the host plant. Seeds were hand collected from a field site in northern Idaho.

AMF species used as pre-inoculants (*Gigaspora margarita* NC121A, *Gigaspora gigantea* NC150A, *Glomus claroideum* NC106A and *Glomus deserticola* NC302A) were originally isolated from North Carolina and were obtained from the International Culture Collection of Arbuscular Mycorrhizal Fungi (INVAM; <http://invam.caf.wvu.edu/index.html>). We built up inoculum for each isolate in pot cultures to obtain sufficient amounts for experimental purposes. This entailed mixing each isolate with sterile sand in 2 l pots and seeding the mixtures with Sudan grass (*Sorghum vulgare* var. *sudanense*). Sudan grass seeds were commercially pre-coated with a fungicide (Outsidepride.com, Inc., Salem, OR) which was removed by soaking in H<sub>2</sub>O for 16 h with periodic rinsing. Plants were grown under glasshouse conditions with weekly additions of 1/8 Hoagland's solution to prevent nutrient limitations. After the plants had seeded the pots were placed in the dark and allowed to dry. The presence of spores corresponding to each AMF species was evaluated using microscopy. Sand and fine root materials from each pot were mixed and used as pre-inoculant materials in subsequent experimental procedures.

### 2.2. Experimental setup

Treatments included pre-inoculation with undiluted sand and fine root materials containing each of the four AMF species described above and a no-AMF treatment in which seedlings were pre-grown in a combined inoculation mixture that had been autoclaved. We created an AMF-free microbial wash to help ensure that non-AMF microbial communities were similar across pre-inoculation treatments. To prepare this wash, each of the four AMF inoculants (25 g) were combined and mixed with 400 ml water using a blender. Mixtures were allowed to settle for 20–30 min and decanted through Whatman No. 1 filter paper (11 µm) to retain AMF infectious propagules while letting bacteria and other non-AMF microbes pass.

Seeds were germinated by placing them on wet filter paper under ambient light. After germination, two seedlings were planted in 15 ml

tubes containing pre-inoculant materials to which 10 ml of the microbial wash had been added. The tubes were placed in a growth chamber (18 h (325 µmol m<sup>-2</sup> s<sup>-1</sup>) light/day; 21 °C; 50–70% relative humidity) and watered every other day with tap water. After seedling emergence, plants in tubes where both seedlings survived were thinned to a single individual per tube by hand pulling.

After 28 days leaf numbers and the longest leaf length of each plant were recorded to provide a non-destructive measure of seedling response to the different pre-inoculation treatments. Plants and pre-inoculation materials were then carefully removed from the initial growth pots by gentle tapping to minimize root system disturbance and transplanted intact into 656 ml D40 Deep-pots (Stuewe and Sons Inc., Oregon) containing field soil homogenized by repeated passage through a 2 mm sieve and hand mixing. The field soil used (cobbly loam Argixeroll) has been previously described in regards to texture and other properties (Lutgen et al., 2003). Prior to transplanting, soil chemical characteristics were analyzed at the University of Idaho Analytical Sciences Laboratory. These analyses included pH (in water; 7.3), NO<sub>3</sub>-N (9.9 µg g soil<sup>-1</sup>), NH<sub>4</sub>-N (7.5 µg g soil<sup>-1</sup>) and Olsen P (7.5 µg g soil<sup>-1</sup>).

After transplanting, plants were grown for an additional 75 days under the same conditions as above except that plants were watered every third day. Each AMF pre-inoculation treatment was replicated five times for a total of 25 experimental units.

### 2.3. Harvest

Plants were clipped at the soil surface, dried (64 °C, >48 h) and weighed to determine shoot biomass. Roots in contact with pre-inoculum materials were removed by extracting soil cores having slightly larger diameter than the tubes used for pre-inoculation using a soil corer (2 cm diameter; 10 cm depth) directly down from the plant crown; these materials were discarded since they would directly reflect pre-inoculation. The remaining soil and roots were then harvested from pots, taking care that any roots that may have been directly exposed to the initial inoculation were not included in samples subsequently analyzed. Roots were washed with deionized H<sub>2</sub>O, blotted dry and aseptically cut into pieces of approximately 2 cm length prior to analyses (see below).

### 2.4. Foliar nutrient concentrations

Green leaf materials dried as above were ash-dried in 10% HCl and subsequently analyzed for elemental nutrient contents (N, P, K, Ca, Mg, S, B, Cu, Fe, Mn, Zn and Na) using ICP spectrometry (University of Massachusetts, Soil and Plant Tissue Testing Laboratory).

### 2.5. Percent AMF root colonization

Approximately half the root systems of all samples were cleared in 10% KOH for 1 h at 80 °C, acidified with 1% HCl for 15 min, and then stained with Trypan Blue in lactoglycerol (0.05%). Stained roots were placed in lactoglycerol overnight to remove residual stain, and cut into ≈ 1 cm pieces, which were placed on microscope slides for analysis of fungal features. Percent AMF colonization was measured by the gridline intersect method at 200× magnification (at least 120 intersects per sample) as described by Rillig et al. (1999).

### 2.6. AMF community composition

A root subsample (0.2 g, wet weight) from each plant was placed in a 2 ml microcentrifuge tube containing a single 3 mm tungsten carbide bead (Qiagen, Valencia, CA) and a small amount of sterile

sand. The tubes were frozen ( $-70^{\circ}\text{C}$ ) using liquid  $\text{N}_2$  and subjected to two bead-beating steps (1 min; Geno/Grinder™ 2000, SPEX CentriPrep, Inc.), interspersed with a liquid  $\text{N}_2$  freezing step, to grind root samples to a fine powder. To each sample, 1 ml DNA extraction buffer [2% CTAB, 1% PVPP, 0.1 M Tris (pH 8.0), 1.4 M NaCl, 0.02 M EDTA] was added, mixed and incubated ( $65^{\circ}\text{C}$ ) for 1 h with additional mixing by tube inversion every 10 min. This was followed by addition of 600  $\mu\text{l}$  chloroform/isoamyl alcohol (24:1), mixing and centrifugation ( $12,000 \times g$ ) for 5 min. The top layer (600  $\mu\text{l}$ ) was then placed in a new microcentrifuge tube to which 900  $\mu\text{l}$  5 M guanidine hydrochloride was added. After thorough mixing, solutions were applied to silicon columns (DNeasy Mini Spin Column, Qiagen) and centrifuged ( $6000 \times g$ ) for 5 min to capture the DNA. After passing 300  $\mu\text{l}$  75% ethanol through the columns, DNA was eluted in 100  $\mu\text{l}$  TE buffer.

PCR amplification of root DNA extracts consisted of two PCR rounds, the first employing the “general fungal” primer pair LR1 and FLR2 (Van Tuinen et al., 1998; Trouvelot et al., 1999) and the second using AMF-specific primers FLR3 (5' labeled with the fluor FAM) and FLR4 (Gollotte et al., 2004). The 25  $\mu\text{l}$  reaction mixtures included HotMaster™ Taq DNA polymerase (Eppendorf, Hamburg, Germany), 1  $\mu\text{l}$  root extracted template DNA or PCR product (diluted 1/10 with molecular grade  $\text{H}_2\text{O}$ ) and 10 pmol of each primer. In order to minimize PCR bias sometimes found to be associated with high cycle numbers (Suzuki and Giovannoni, 1996; Uejima et al., 2000), we used 25 cycles for reactions using the primer pair LR1 and FLR2 and 30 cycles for the second, AMF-specific reaction using primers FLR3 and FLR4. Thermal cycling for all reactions included an initial denaturing step of  $95^{\circ}\text{C}$  for 5 min, 25 or 30 cycles as described above consisting of 1 min at  $95^{\circ}\text{C}$ , 1 min at  $58^{\circ}\text{C}$  and 1 min at  $65^{\circ}\text{C}$ , followed by a final extension step of  $65^{\circ}\text{C}$  for 10 min. Products of these reactions were quantified by image analysis of agarose gels following electrophoresis with Low DNA Mass Ladder (Invitrogen, Carlsbad, CA) as the size standard.

PCR products were then purified using the GenCatch™ PCR cleanup kit (Epoch Biolabs, Inc., Sugar Land, TX) and subsequently digested with the restriction enzymes MboI and TaqI (New England Biolabs, Beverly, MA) in separate reactions. To determine the efficacy of digestion, *E. coli* 16S rDNA PCR amplicons (primers 27f and 536r), 5' end-labeled with the fluor NED, were included in all reactions. Each digestion reaction, containing 1  $\mu\text{l}$  digestion control DNA, 11  $\mu\text{l}$  purified PCR product and 3 U MboI or TaqI in the manufacturer's recommended buffer, was incubated for 3 h at  $37^{\circ}\text{C}$  (MboI) or  $65^{\circ}\text{C}$  (TaqI), followed by enzyme heat inactivation at  $94^{\circ}\text{C}$  for 10 min. All reactions were subsequently treated with Mung Bean endonuclease (3 U; New England Biolabs) to remove single-stranded DNA sequences which are known to produce “pseudo-T-RFs” (Egert and Friedrich, 2003).

T-RF size distributions for each sample were determined using an ABI 3100 automated capillary DNA sequencer (Applied Biosystems, Foster City, CA) with ROX-500 (Applied Biosystems) as the size standard. T-RF size determination and quantification was performed using Genemapper software (Applied Biosystems).

We used the Microsoft Excel macro Treeflap (Rees et al., 2004; <http://www.wsc.monash.edu.au/~cwalsh/treeflap.xls>) to convert each fragment size present in T-RFLP profiles to the nearest integer value and to subsequently align peaks against rounded sizes of the fragments. Total relative fluorescence of T-RFLP profiles derived from each sample were standardized to 5000 relative fluorescence units with a minimum peak height threshold of 50 fluorescence units.

Soil used in this study had previously been extensively characterized in regards to AMF molecular diversity and site-specific methods developed for discriminating between the AMF species and/or ribotypes present (Mummey and Rillig, 2007). These

analyses included cloning and sequencing of PCR amplicons derived from DNA extracted directly from the soil, as well as from roots of different plant species grown in this soil, and showed that the soil contains a relatively broad diversity of *Glomus* group A and *Glomus* group B representatives. Simulated digestion of cloned rRNA gene sequences with a range of restriction endonucleases was performed using the computer program TRFSEQ. This allowed for enzyme selection to be optimized for discrimination via T-RFLP analysis of all ribotypes present. From these analyses it was concluded that two restriction endonucleases (MboI and TaqI) meet criteria for optimal T-RFLP discrimination of AMF present in this soil (Mummey and Rillig, 2007). Simulated digestion also provided information pertaining to T-RF sizes associated with all ribotypes present in our database. These were compared to T-RF sizes present in T-RFLP profiles, allowing for matching of fragment sizes in T-RFLP profiles of each sample with phylogenetically classified sequences. We considered a phylotype to be present in a sample if T-RF size matches to database sequences were found in T-RFLP profiles after digestion with both TaqI and MboI (Table 1; Supplementary Material).

## 2.7. Data analyses

Differences in plant shoot biomass, leaf number and leaf length, foliar nutrients, AMF percent root colonization and AMF phylotype numbers identified by T-RFLP analysis between treatments were examined by one-way ANOVA using the statistical program SPSS (v15.0). For comparison of individual means we used Tukey honestly significantly different tests. ANOVA assumptions pertaining to homogeneity of variance were evaluated by calculating Levene's statistics (SPSS).

We tested for the influence of pre-inoculation treatments on AMF communities subsequently colonizing roots by using distance-based redundancy analysis (db-RDA; Legendre and Anderson, 1999) constrained by pre-inoculation treatment identities. Bray–Curtis similarity matrices were constructed for phylotype presence or absence data and used to compute principal coordinates using the computer program PrCoord (Canoco software; ter Braak and Šmilauer, 1998). Monte Carlo permutation tests (999 iterations) were performed to assess the significance of canonical axes showing relationships between AMF community data of each pre-inoculation treatment group.

## 3. Results

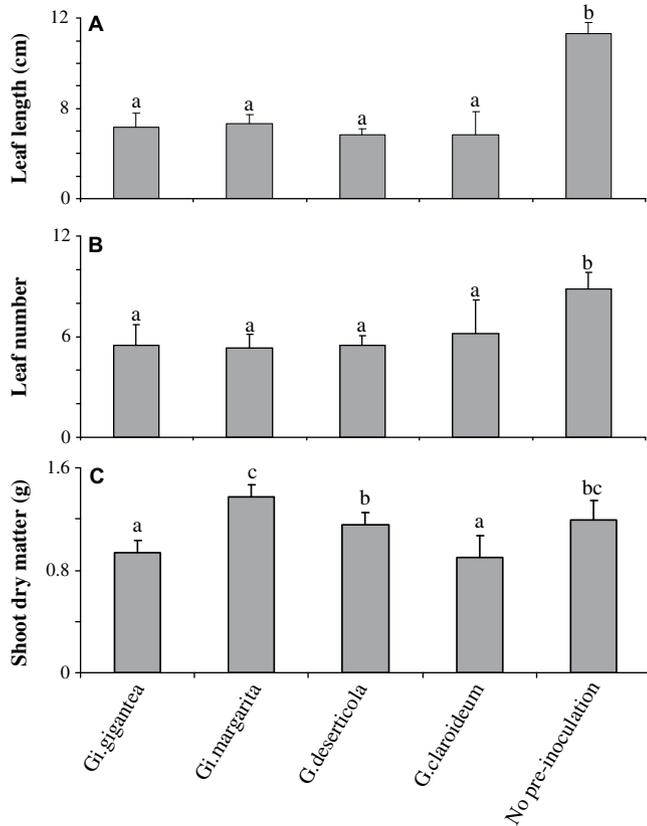
### 3.1. Leaf lengths and numbers before transplanting

Both leaf numbers and longest leaf lengths measured before transplanting of pre-inoculated plants were significantly greater for plants not pre-inoculated with AMF (Fig. 1). No significant

**Table 1**

Phylogenetic groups as depicted in Fig. 8, family and species level associations, T-RF sizes yielded after digestion with MboI and TaqI, and GenBank accession numbers of representative cloned sequences derived from the study soil and that were used for identification of phylotypes via T-RF size matching.

Group	Family (species)	MboI	TaqI	Accession
G1	<i>Glomus</i> A ( <i>G. mosseae</i> )	107	185	DQ677407, DQ677441
G5	<i>Glomus</i> A ( <i>G. intraradices</i> )	186	184	DQ677389, DQ468710
G23	<i>Glomus</i> A	370	149	DQ677429, DQ677420
G24	<i>Glomus</i> A ( <i>G. microaggregatum</i> )	155	50	DQ677409, DQ677463
G25	<i>Glomus</i> A	189	50	DQ468812, DQ677468
G6	<i>Glomus</i> A	151	50	DQ468807, DQ468809
G29	<i>Glomus</i> B ( <i>G. claroideum</i> )	369	54	DQ677418, DQ677427
G31	<i>Glomus</i> B	59	47	DQ677417, DQ677455



**Fig. 1.** Comparison of longest leaf lengths (A) and leaf numbers (B) for plants subjected to different AMF pre-inoculation treatments. Leaf length and number measurements were taken before plants were planted to whole-soil, mixed-AMF community inoculum. Shoot biomass (C) was measured at the end of the experiment. Error bars represent standard deviation. Different letters signify significant differences ( $\alpha = 0.05$ ).

differences between plants pre-inoculated with different AMF species were found for these measures indicating that the initial cost of the symbiosis to the plant was similar across pre-inoculation treatments.

### 3.2. Final shoot biomass

Shoot biomass of plants pre-inoculated with different AMF species was found to be significantly different overall, with *Gi.*

*margarita* pre-inoculation yielding the highest values (Fig. 1). Shoot biomass of plants pre-inoculated with *Gi. gigantea*, *G. deserticola* and *G. claroideum* were significantly less ( $p < 0.05$ ) than what was found for plants pre-inoculated with *Gi. margarita*. Shoot biomass of plants pre-inoculated with *G. claroideum* or *Gi. gigantea* were significantly less than all other treatments.

### 3.3. Foliar nutrients

Analysis of foliar nutrients indicated that pre-inoculation with *Gi. margarita* resulted in significantly greater leaf P and Cu concentrations than treatments in which plants were pre-inoculated with *Gi. gigantea* or *G. claroideum* (Fig. 2). Leaf P or Cu contents were not significantly different between any other treatments. A strong correlation was found between leaf P and Cu concentration across all samples ( $R = 0.92$ ,  $p < 0.005$ ).

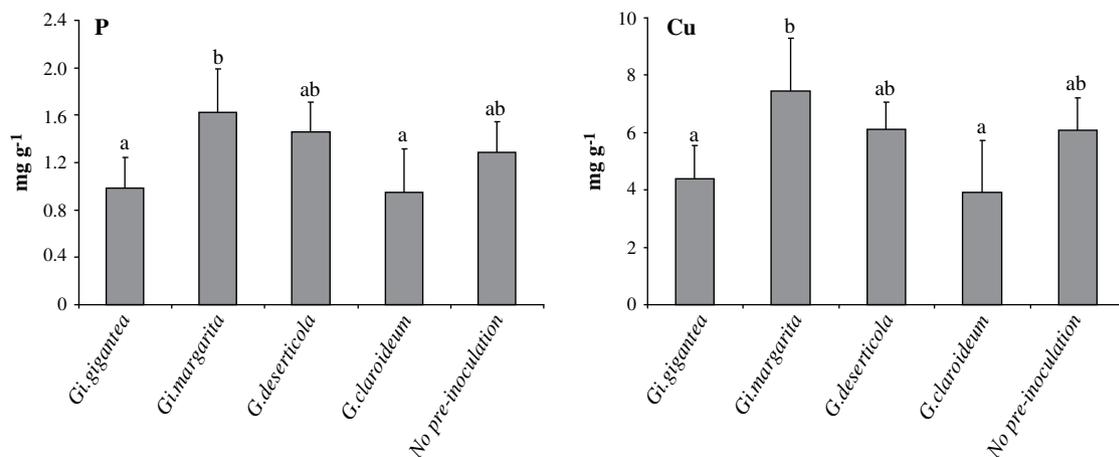
Leaf zinc concentrations were found to be significantly greater in plants either not pre-inoculated with AMF or pre-inoculated with *G. claroideum* than for plants pre-inoculated with *G. deserticola* (data not shown). No other significant differences were found for leaf zinc concentration between treatments. No significant differences between treatments were found for any of the other nutrients analyzed (data not shown).

Comparison of nutrient uptake, calculated by multiplying nutrient concentration by shoot biomass, indicated that plants pre-inoculated with *Gi. margarita* acquired significantly greater P and Cu from the soil than plants of all other pre-inoculation treatments (Fig. 3). Conversely, plants pre-inoculated with *Gi. gigantea* and *G. claroideum* contained significantly less total P and Cu than plants not pre-inoculated with AMF (Fig. 3).

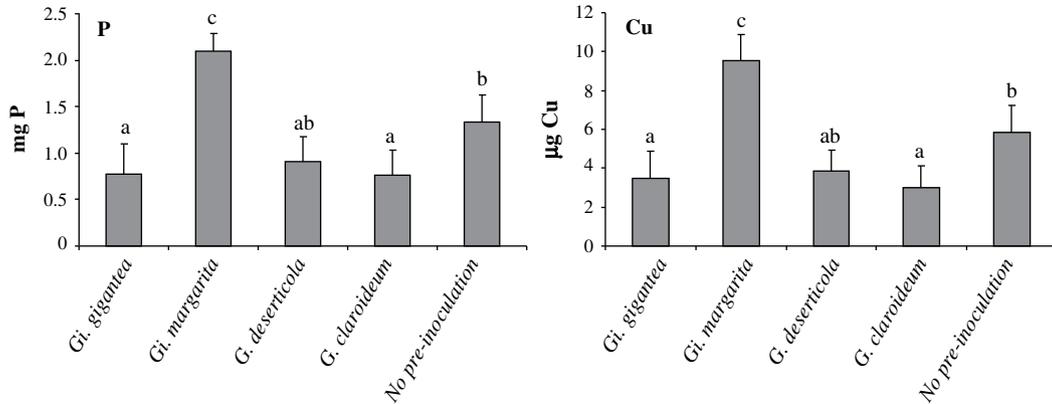
### 3.4. Percent AMF root colonization

To determine the efficacy of pre-inoculation treatments, roots were sampled immediately prior to transferring each plant to field soil and AMF root colonization assayed by microscopy as described above. Although root materials were insufficient for accurate calculation of AMF percent root colonization for all samples, these analyses indicated that roots of all AMF pre-inoculation treatments were colonized, whereas root colonization by AMF was absent in samples not exposed to AMF pre-inoculants.

Percent AMF root colonization at the end of the experiment averaged 34% (SD = 0.10) across all treatments; however, no



**Fig. 2.** Foliar P and Cu contents as determined by ICP analysis. Error bars represent standard deviation. Different letters indicate significant differences between pre-inoculation treatments ( $\alpha = 0.05$ ).



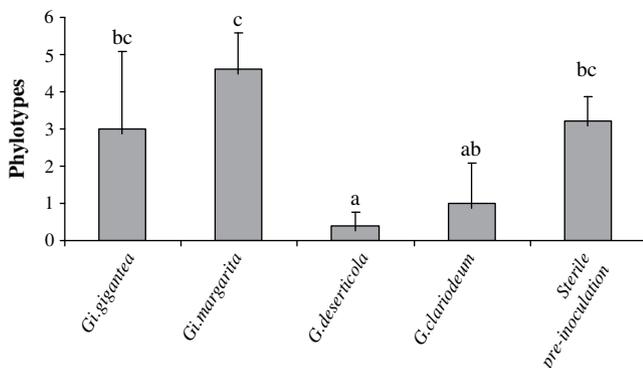
**Fig. 3.** Comparison of total P and Cu in shoots of plants from different pre-inoculation treatments. Error bars represent standard deviation. Different letters indicate significant differences ( $\alpha = 0.05$ ).

significant differences ( $\alpha = 0.05$ ) between treatments were found (data not shown).

### 3.5. AMF communities in roots at final harvest

Pre-inoculant identity was found to significantly influence AMF phylotype richness detected in root samples ( $p < 0.005$ ), with *G. deserticola* and *G. claroideum* pre-inoculation treatments yielding the lowest ribotypes numbers (Fig. 4). After removal of T-RF sizes anticipated for AMF pre-inoculants, three *G. deserticola* and two *G. claroideum* pre-inoculation samples yielded no additional T-RF sizes.

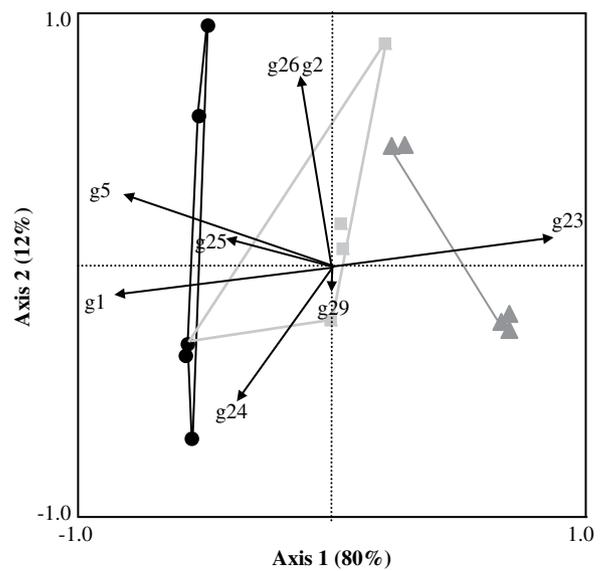
Distance-based redundancy analysis of AMF communities assembled after *Gi. gigantea*, *Gi. margarita* and sterile pre-inoculation treatments (*G. deserticola* and *G. claroideum* pre-inoculation treatments were excluded from the analysis due to lack of informative characters as mentioned above) indicated that pre-inoculant identity accounted for a significant amount of the variance in the AMF ribotypes identified by matching T-RF sizes with our database sequences (Trace = 0.658,  $F$ -ratio = 11.542,  $p < 0.002$ ). Moreover, graphical depiction of principle coordinates shows clear separation of AMF communities assembled after *Gi. gigantea*, *Gi. margarita* and sterile pre-inoculation treatments (Fig. 5). Similar analyses comparing *Gi. gigantea* and *Gi. margarita* pre-inoculum treatments indicated that pre-inoculant species identity had a significant influence on AMF community composition that assembled within roots (Trace = 0.442,  $F$ -ratio = 6.328,  $p = 0.042$ ).



**Fig. 4.** Comparison of the number of different AMF phylotypes identified in roots of each pre-inoculation treatment via T-RFLP analysis. Error bars represent standard deviation. Different letters signify significant differences between treatments ( $\alpha = 0.05$ ).

## 4. Discussion

Our results indicate that pre-inoculation of seedlings with AMF can significantly influence the richness and diversity of the resident AMF communities subsequently assembling in roots after planting in whole-soil, mixed-AMF community inoculum. In the case of pre-inoculation with *G. deserticola* or *G. claroideum*, ribotype numbers of the native AMF were significantly reduced, in some root samples to below detection limits (Fig. 4). This result has important implications for use of AMF pre-inoculants, as such reductions in AMF diversity could compromise the ability of the community to provide services. In light of recent evidence that AMF community richness influences plant communities (e.g. van der Heijden et al., 1998), these results could also have broad implications for plant diversity and productivity if non-indigenous AMF species are shown to achieve dominance beyond the inoculated plant.



**Fig. 5.** Ordination plot depicting relationships between AMF phylotypes associated with *Gi. gigantea* (●), *Gi. margarita* (■) and sterile pre-inoculation (▲) treatments. *Glomus* pre-inoculation treatments are not presented because only T-RF sizes matching pre-inoculants were present in multiple samples. Vector arrows depict the relative importance of different phylotypes to group separation (see Table 1; Supplementary Material). Overlapping symbols were shifted slightly for data visualization. Lines connecting sample symbols are presented only as an aid for visual delineation of treatment groups. Numbers in parentheses associated with each axis indicate the amount of variance explained by principal coordinate analysis.

In contrast to pre-inoculation with *G. deserticola* or *G. claroidesum*, pre-inoculation with Gigasporaceae isolates, irrespective of the species used, resulted in AMF ribotype numbers similar to those of plants that were not pre-inoculated. A possible explanation for this observation may be differences in colonization strategies typically observed between the Gigasporaceae and the Glomeraceae (Hart and Reader, 2002; Maherali and Klironomos, 2007), the latter of which dominate the study soil (Supplementary Material; Mummey and Rillig, 2008). Since the majority of Gigasporaceae biomass is typically located in hyphae outside of roots, competition between the *Gigaspora* pre-inoculants and the resident AMF of our study soil may be less pronounced than for the *Glomus* pre-inoculants, thus allowing increased diversity to colonize the roots. Our results suggest that pre-inoculation with *Gigaspora*, at least in our experimental system, has less potential to compromise the AMF community richness than *Glomus* pre-inoculation.

A number of studies have shown that AMF inoculation does not always result in increased benefits to plants (e.g. Johnson et al., 1997; Requena et al., 2001; Klironomos, 2003). Our results show differential plant responses to pre-inoculant identity. This was especially pronounced in regards to P and Cu acquired in plant biomass (Fig. 2). In the case of *Glomus* pre-inoculants, not only did this result in significantly reduced AMF ribotype diversity, but these treatments exhibited low shoot biomass and tissue P and Cu concentrations. Perhaps by decreasing the diversity of AMF species subsequently able to colonize the plant pre-inoculation with *Glomus* decreased the relative benefit of the symbiosis to the host plant (Jansa et al., 2008).

Even though AMF ribotype diversity was relatively high in plants pre-inoculated with *Gigaspora* relative to *Glomus*, very different plant responses were found for the two *Gigaspora* pre-inoculant treatments. Pre-inoculation with *Gi. margarita* resulted in significantly greater shoot production than all treatments except the pre-inoculation treatment lacking AMF (Fig. 1C). Plants pre-inoculated with *Gi. margarita* also acquired significantly greater P and Cu in their shoot biomass. In contrast, plants pre-inoculated with *Gi. gigantea* were among the lowest of any treatment in regards to shoot biomass, as well as P and Cu acquisition. This indicates that the overall costs of the symbiosis to the plants were quite different for the two *Gigaspora* pre-inoculation treatments.

Although AMF ribotype diversity was high in roots pre-inoculated with either of the two *Gigaspora* spp., the composition of the AMF communities assembled differed significantly. This suggests that differential benefits to the plants could be driven not only directly by cost–benefit relationships between the plants and AMF inoculants, but indirectly by alteration of the AMF communities subsequently assembled after pre-inoculation. Presuming that both *Gigaspora* spp. are similar in their root colonization strategies, these results further suggest that other mechanisms besides differences in colonization strategy, such as alteration of root exudate composition (Piniór et al., 1999; Vierheilg et al., 2000; Scervino et al., 2005) or differential impacts on other components of the soil microbial community (Rillig et al., 2006), are driving AMF community assembly.

From a horticultural standpoint, our results indicate that nursery-applied AMF can suppress colonization with indigenous, beneficial AMF species. The degree to which this could compromise the ability of the AMF community of a given site to provide beneficial services over time is unknown and should be the focus of further studies. The apparent differences in AMF species assembled after different pre-inoculations, however, also suggests that it may be possible, via rational selection of pre-inoculants, to exclude specific AMF species with negative effects. Manipulation of AMF community composition in this way may provide the means to enrich AMF species providing services of interest while decreasing

the importance of less beneficial species. If exclusion of specific AMF species occurs, or if AMF community assembly is otherwise altered, then at least the potential exists for rational manipulation of AMF community assembly to influence functional relationships between host and AMF symbionts. This may be especially important in light of the trend towards less intensive agricultural practices that rely less on chemical inputs that replace AMF functions in agroecosystems (Gosling et al., 2006). Pre-inoculation with specific AMF species may be a viable strategy for increasing AMF services (Sorensen et al., 2008).

Our results suggesting that AMF inoculation has the potential to diminish resident AMF communities could be important when planting into a rich AMF community. On the other hand, in cases where the resident AMF community is impoverished, positive plant responses to inoculants may actually benefit resident AMF communities. Using an agricultural soil, Antunes et al., in press tested for the comparative effects of disturbance and AMF inoculation and found that the former had important effects on AMF community composition, while inoculation did not. The authors also demonstrated that the inoculant species (*G. intraradices*) seemed to act synergistically with the resident AMF community under disturbed conditions. Although further research is required using additional plant species and soils with different degrees of AMF diversity to determine the extent to which our results can be generalized, our results indicate that, depending on the AMF used, pre-inoculation can have a significant influence on how AMF communities are assembled in roots over short time scales.

Our results suggesting that establishment priority can be an important determinant of AMF community structure may have important implications beyond pre-inoculation practices. Given that a number of factors can result in a plant being exposed to different subsets of the AMF community throughout its life cycle [e.g. the identity of neighboring plant species and their phenology (Pringle and Bever, 2002; Mummey and Rillig, 2006; Hawkes et al., 2006), AMF growth rate seasonality (Husband et al., 2002a,b; Pringle and Bever, 2002) and AMF community small-scale spatial variability (Mummey and Rillig, 2008)], more research is clearly needed to elucidate the importance of establishment priority to AMF community structure. However, the results of this study clearly indicate that the structure of AMF communities that subsequently establish in a pre-inoculated host plant depends on pre-inoculant identity.

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## Appendix A. Supplemental material

Supplementary information for this manuscript can be downloaded at doi: 10.1016/j.soilbio.2009.02.027.

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