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Microbiota accompanying different arbuscular mycorrhizal fungal isolates influence soil aggregation

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Summary

Arbuscular mycorrhizal fungi (AMF) are key components of ecosystems through their influence on plant communities and ecosystem processes. A major source of information regarding the importance of AMF species richness on process rates are mesocosm experiments using different levels of diversity of AMF as provided by single-species cultures of AMF. Since AMF inocula are generally made available in the form of non-sterile pot culture material, it is possible that AMF symbiosis-associated microbiota are at least partially responsible for some effects hitherto directly attributed to the AMF mycelium. Here, we provide evidence that microbiota associated with single-species cultures of AMF (after long-term pot culture enrichment of 7–8 years) can strongly affect the ecosystem process of soil aggregation. This effect occurred in an AMF isolate specific manner, but in the absence of live and active AMF mycelium. We additionally documented large differences in microbiota communities associated with the different AMF inocula (using PLFA analyses), suggesting that these differences were at least partly responsible for the observed changes in soil aggregation. This result points to AMF–microbiota interactions as a largely unexplored mechanism underlying soil aggregation (and potentially other ecosystem processes). We suggest that a reinterpretation of previous experiments using greenhouse-derived AMF cultures may be necessary, and the need to consider AMF symbiosis-associated microbiota in mechanistic studies of AMF and mycorrhizae in general is emphasized.

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Introduction

Arbuscular mycorrhizal fungi (AMF) are symbionts associated with the roots of most terrestrial plants. The presence of these fungi has been shown to have a large influence on the structure of plant populations (Lu and Koide, 1994; Philip et al., 2001) and communities (Hart et al., 2003) as well as on ecosystem processes (Rillig, 2004a), such as soil aggregation (Tisdall and Oades, 1982) and nutrient cycling (Read and Perez-Moreno, 2003).

Recent studies have also shown that there is wide variation in the life history strategies of AMF species (Hart and Reader, 2002), and that AMF species can differ greatly in their abilities to influence plant productivity (Klironomos, 2003). Furthermore, studies that have manipulated the make-up of AMF communities have shown that not only does the presence of AMF but also the composition and diversity of AMF communities influence the structure and functioning of ecosystems (van der Heijden et al., 1998). As a result, there is concern that disturbances that can reduce the diversity of AMF, such as agricultural practices (Jansa et al., 2003; Oehl et al., 2003) or factors of global change (e.g., Egerton-Warburton and Allen, 2000), can indirectly affect ecosystem functioning.

One potential problem with AMF diversity manipulation experiments, however, is the difficulty in distinguishing effects of AMF mycelia from those of associated microbes. There are a number of microbes that grow in association with AMF mycelia and spores (e.g. Mansfeld-Giese et al., 2002), as well as in the rhizosphere of culture host plants, and typical AMF culturing methods do not filter out these associated microbes. AMF are obligate biotrophs, so the main method for culturing these fungi is in association with a host plant under non-sterile greenhouse conditions. This is how most AMF isolates are distributed by researchers and by international culture collections of AMF (e.g., INVAM). Soil biota also contained in these cultures would generally be regarded as "background", and there is an implicit assumption in AMF diversity studies (which use such cultures) that there are no functional differences among AMF species inocula in the communities of associated microbiota. However, this assumption has not been properly tested. Furthermore, if these accompanying microbes are also effective in performing certain ecosystem functions generally attributed to AMF, previous studies may have erroneously interpreted these as resulting from the direct action of the AMF mycelia.

In the present study, our goal was to test the hypothesis that AMF associated microbes have distinct roles in ecosystem processes that at least partly account for effects hitherto directly attributed to the mycelia of the different AMF species. We have focused on one ecosystem process, soil aggregation. Soil structure is an ecosystem parameter of great importance, influencing a variety of biogeochemical processes (such as carbon storage; e.g., Jastrow, 1996) and the growth of soil biota (e.g., Rillig and Steinberg, 2002). Observational and correlational approaches, (Jastrow et al., 1998; Rillig et al., 2002a), and experimental approaches, employing compartmentalized pot designs separating AMF mycelium from root influences (Thomas et al., 1993), have emphasized the paramount role of AMF in soil aggregation. However, little is known regarding the role of different AMF isolates in this process (Schreiner et al., 1997), and still less about the potential importance of AMF-associated microbes.

Materials and methods

AMF cultures

Culture substrate of single species, naturally co-occurring AMF from the Long-Term Mycorrhiza Research Site (LTMRS) at the University of Guelph (Guelph, Ont., Canada) were used in this study. The isolates used represented several genera, and were *Glomus etunicatum*, *Glomus* sp.2, *Acaulospora morrowiae*, *Entrophospora columbiana*, *Gigaspora margarita*, and *Scutellospora heterogama* (Klironomos, 2003). These cultures were grown 7–8 years in the greenhouse under standardized conditions, going through rotations of sterilized soil, perlite or turface media with leek as the host plant. For each culture cycle, a quarter of the contents (including other microbiota, roots, and AMF propagules) of one pot culture (1 or 2 L pots) was used to establish a new generation of cultures. For the last culture cycle, yielding the material used here, infective propagules of all species of AMF were grown in perlite substrate. After 5 months, cultures were harvested, dried, roots were chopped into small fragments, and the pot contents were placed in plastic bags for use in this study.

AMF culture material characterization

In order to quantify potential covariates for changes in water stability of aggregates in response to adding inoculum material, we needed to measure attributes of the microbiota contained in the inoculum. Hyphal lengths are not necessarily a

good predictor of AMF-associated bacterial populations (Olsson et al., 1996; Andrade et al., 1998), and since there were also root fragments contained in the inoculum, we characterized microbial populations in the original inoculum using phospholipid fatty acid analysis (PLFA). PLFAs were extracted (from two replicate 30 g samples of inoculum for each species) and analyzed according to White and Ringelberg (1998). Briefly, lipids were removed from samples into chloroform using a modified Bligh and Dyer (1956) extraction procedure. Phospholipids were separated from other lipids by silica acid chromatography and derivatized to their fatty acid methyl esters (FAMEs) for analysis by gas chromatography. FAMEs were identified by relative retention times, co-elution with purchased standards, and comparison of samples between capillary columns of differing polarity (HP-5 (crosslinked 5% phenal methyl silicon) $50\,m \times 0.32\,mm \times 0.52\,\mu m$ film, HP-225 (50% CNPrPh Me Siloxane) $30\,m \times 0.32\,mm \times 0.25\,\mu m$ film). Final verification of peak identity was made by gas chromatography/mass spectroscopy on representative samples. FAMEs were quantified on a HP-225 column using a HP 6890 series GC system and protocol according to Frostegård et al. (1993). PLFAs were summed per sample to obtain a total amount of nmol PLFA g^{-1} inoculum, as an indication of total microbial biomass. AM fungi themselves produce a variety of fatty acids (Graham et al., 1995; Bentivenga and Morton, 1996); any fatty acids of fungal origin were excluded before carrying out microbial community analyses to avoid confounding effects.

In order to ascertain whether potential differences in microbiota abundance associated with the different AMF cultures are consistent over time (or unique to the culture cycle selected for this study), we also report on bacterial biomass from culture cycle 15. The experiment reported here was conducted with material from culture cycle 21, i.e. six pot culture cycles (>1 yr) later (each culture cycle lasted 2–3 months). Bacterial biomass (mg kg^{-1} culture material; $n = 5$ pots) for culture cycle 15 was measured using the europium (III) thenoyltrifluoroacetonate staining method as described in Morris et al. (1997), whereas it was measured for culture cycle 21 using PLFA. Since europium staining and PLFA are two very different methods, we only used correlation analysis to compare trends in results.

Greenhouse experiment

Soil (0–30 cm depth) from the North Hills field site in Missoula, Montana (see Lutgen et al., 2003 for soil characteristics) was used to grow the different AMF species in a greenhouse at University of Montana, Missoula. Field-collected soil was moistened and heat treated at 88 °C for 8 h, then dried at 80 °C overnight to further reduce populations of native soil microbiota and to eliminate AMF contained in the soil. Heat-treated soil was sieved (4 mm), crushed to reduce average aggregate stability (1–2 mm size class) from ca. 80% to 40% (as determined in preliminary tests), and then sieved to 2 mm. Aggregate stability was reduced to maximize our ability to detect changes in that variable.

This prepared soil was filled into pots (440 g), thoroughly mixed together with 20 g of inoculum from the various AMF species, or with an equal amount of sterile perlite for the control treatment. Pots were seeded to *Bromus inermis*, a grass that is dominant at the LTMRS (Klironomos et al., 2000).

In order to eliminate colonization of roots by AMF inocula that were added to the pots containing previously heat-treated soil (see above) we applied a heat pulse 1 d after addition of inoculum to pots of 45–50 °C lasting for 1 d (temperature measured in the center of pots). Thus, no viable AMF inoculum should have been present in the experiment. In a separate set of control pots, grown at the same time, without the initial heat treatment, but using the same inoculum sources, AMF isolates colonized roots of *B. inermis* satisfactorily (data not presented; these pots were not used further in this study). Heat-inactivating AMF was used as a strategy to avoid having to extract microbiota from the culture medium; using this design all microbiota were transferred to the experimental pots. We made the assumption that microbiota other than temperature-sensitive AMF would be able to colonize the soil in the pots, and a caveat of this design is that some microbial populations would have been reduced through the temperature treatment as well.

Pots were watered as needed to avoid water stress, and were adjusted to three plants each one week after seeding. Positions of pots on the greenhouse bench were re-randomized every few weeks until harvest (after 9 weeks).

Post-harvest analyses

At harvest, shoots and roots were separated, dried (60 °C) and weighed. After weighing, roots were cleared and stained with Trypan Blue for AMF, and root colonization was examined at $200\times$ magnification as described in Rillig et al. (2002b). Soils were air-dried (50 °C), and sieved to

retrieve the 1–2 mm aggregate size class (for which the soil preparation was optimized). For this size class of aggregates we measured water stability after capillary rewetting (10 min) after Kemper and Rosenau (1986), using a wet-sieving machine. Soil and aggregate weights were adjusted for coarse matter (>0.25 mm). For the 1–2 mm size fraction we also measured hyphal length, using an aqueous filtration extraction method as described in Rillig et al. (2002b). Hyphae of AMF origin were distinguished from others by a suite of morphological characteristics at 200 × magnification as described in Rillig et al. (2002b). Glomalin-related soil protein (GRSP), a fraction of soil organic material important in soil aggregation (Wright and Upadhyaya, 1998) that is linked to AMF origin (Rillig, 2004b), was extracted and quantified with a Bradford assay according to Rillig et al. (2002b).

Statistical analysis

We used ANOVA and ANCOVA to test for differences among treatments. Total PLFA mass (nmol PLFA g^{-1} inoculum) was used as a covariate to test if differences in initial inoculum microbial biomass could account for observed differences in water stability. After ANOVA, means were separated using Tukey–Kramer HSD, or comparisons with control treatments were carried out with Dunnett's test (JMP software, SAS Institute). Where comparison among treatments containing AMF was the main goal (e.g., for water stability), the control treatment was excluded from the analysis. For all tests we examined normal distribution of residuals and homogeneity of variances.

We performed a principal component analysis (PCA) on mole-percentages of different PLFA species to capture overall microbial community differences among samples, using only the 30 most common PLFAs that were present in all samples. Importantly, this procedure avoided including zero mole-percentages, which could lead to biased comparisons that are possible when using high and low biomass samples. PCA scores were calculated using the correlation matrix with the NCSS 2001 software (Kaysville, UT, USA).

Results

Inoculum characterization

Total amount of PLFA (nmol PLFA g^{-1} inoculum) differed significantly among the AMF species inocula (ANOVA; $n = 2$; $F = 56.1$; $P < 0.0001$). The *Gigaspora* and *Scutellospora* isolates had the lowest abundance of total PLFA in their inocula, and the *Entrophospora* had the highest (Table 1). PLFAs indicative of bacteria (i.e. excluding any PLFAs of AMF or other fungal origin) showed a similar pattern as total PLFA (Table 1), and there were also significant differences among AMF-isolates (ANOVA; $n = 2$; $F = 59.8$; $P < 0.0001$). Bacterial biomass from culture cycle 15 (derived from microscopic counts; Table 1) was also significantly different among AMF species inocula (ANOVA; $n = 5$; $F = 20.2$; $P = 0.0001$), and was highly correlated with bacteria-indicating PLFAs from culture cycle 21 (the one used in the present experiment) (linear regression using log-transformed data; $r^2 = 0.81$; $n = 6$ [for six AMF species]; $P = 0.01$).

PCA of inoculum microbial communities, using only non-zero PLFA mole-percentages and excluding any PLFAs of fungal origin, showed that microbial communities contained in different AMF isolate cultures clearly diverged (Fig. 1).

Table 1. Mean total PLFA amount, bacterial PLFA total amount for different AMF initial inocula

AMF isolate	PLFA total (nmol PLFA g^{-1})	PLFA bacteria (nmol PLFA g^{-1})	Bacterial biomass (mg kg^{-1}) from culture cycle 15
Glomus etunicatum	14.85 (0.85)^{c}	6.22 (0.26)^{c}	3.53 (0.8)^{bc}
Glomus sp. 2	15.75 (0.05)^{c}	6.23 (0.16)^{c}	4.74 (0.6)^{a}
Acaulospora morrowiae	23.30 (2.8)^{b}	9.60 (1.12)^{b}	4.97 (0.7)^{a}
Entrophosphora columbiana	31.45 (0.75)^{a}	13.10 (0.42)^{a}	4.46 (1.1)^{ab}
Gigaspora margarita	6.55 (0.45)^{d}	2.57 (0.18)^{d}	2.27 (0.7)^{cd}
Scutellospora heterogama	7.35 (0.65)^{d}	2.81 (0.31)^{d}	1.97 (0.6)^{d}

Shown are means, standard error in brackets ($n = 2$); also bacterial biomass from culture cycle 15 (culture 21 was used in this study as inoculum) shown for comparison with bacterial PLFA data ($n = 5$ for bacterial biomass). Means (standard errors in brackets) followed by the same letter (within a column) are not significantly different at $P < 0.05$ (Tukey–Kramer HSD).

Absence of AMF growth in the experiment

In order to demonstrate that viable AMF mycelium did not have a direct influence on the soil aggregation responses observed, we measured three response variables: percent root colonization, hyphal length, and GRSP concentration (the latter two in the 1–2 mm size class, which is the one for which soil aggregation was measured). We did not detect any colonization of roots by AMF (data not shown). AMF hyphal lengths measured on 1–2 mm size aggregates were significantly lower in the treatments that received AMF inocula and their associated microbiota (except for *A. morrowiae*, which showed the same trend) compared to the control treatment, which was also planted, but received only sterile perlite as inoculum (Table 2). Since in the sterile treatment no microbiota were added, little decomposition occurred (as tested in a preliminary study, data not shown). Likewise, concentrations of GRSP were also significantly decreased in the treatments receiving AMF inocula (except for *G. etunicatum* inoculum, which showed a similar trend) (Table 2), indicating decomposition by the introduced microbial communities contained in the AMF inocula.

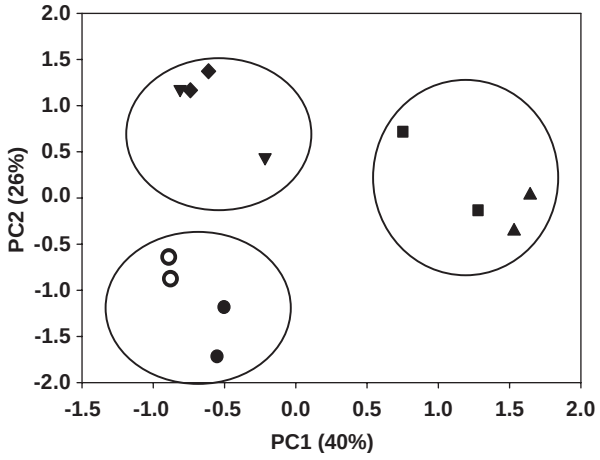

Figure. 1. Principal component analysis (using only PLFAs of non-fungal origin) showing differences in microbial community structure among the different AMF inocula (from culture cycle 21). Symbols: filled circle = *E. columbiana*, triangle up = *Gi. margarita*, square = *S. heterogama*, triangle down = *G. etunicatum*, diamond = *Glomus species 2*, open circle = *A. morrowiae*. Large circles indicate distinct groupings of communities.

Plant growth

Final harvest of plant material revealed that there were no significant differences in *B. inermis* shoot or root weight compared to the control treatment, or within the treatments that received AMF inocula (Table 3). Lack of plant effects led to the exclusion of these variables as covariates in statistical models testing for effects of inocula on soil aggregate water stability.

Soil aggregate water stability

Water-stability of soil aggregates in the 1–2 mm size class ($WSA_{1-2\,mm}$; in experimental soils approx. 12% of total soil weight data not shown) are presented in Fig. 2. Compared to the control treatment, the treatments receiving microbial communities from the different AMF inocula overall had a significantly higher $WSA_{1-2\,mm}$ (linear contrast, $P<0.05$). Examining individual AMF inocula treatments, all except *Gigaspora margarita* had significantly higher $WSA_{1-2\,mm}$ compared to the control (Dunnett's test, $P<0.01$).

Subsequently, we were interested in comparing effects only within the treatments that received AMF inocula. ANOVA revealed significant

Table 2. End-of-experiment assessment of mycorrhizal parameters for the 1–2 mm size class of soil aggregates

AMF inoculum	AMF hyphal length ($m\,g^{-1}$ aggregates)	GRSP concentration (mg Bradford reactive protein g^{-1} aggregates)
Control (plant only; no AMF)	1.20 (0.05)	11.24 (0.16)
Glomus etunicatum	0.726 (0.089)**	9.29 (1.30) NS
Glomus sp. 2	0.845 (0.095)*	4.48 (0.68)**
Acaulospora morrowiae	0.917 (0.102) NS	6.15 (1.24)**
Entrophospora columbiana	0.851 (0.095)*	6.21 (1.34)**
Gigaspora margarita	0.726 (0.092)**	4.81 (0.59)**
Scutellospora heterogama	0.854 (0.092)*	5.14 (0.57)**

Shown are means, standard errors ($n = 5$; except for *E. columbiana*, $n = 4$), and significance of comparisons with control treatment (Dunnett's test; NS = not significant; $^{*}P<0.05$, $^{**}P<0.01$).

Table 3. Root and shoot weights of *Bromus inermis* plants at final harvest

AMF species inoculum	Shoot mass ($g\,pot^{-1}$)	Root mass ($g\,pot^{-1}$)
Control (plant only; no AMF)	0.367 (0.051)	0.344 (0.063)
Glomus etunicatum	0.212 (0.047)	0.306 (0.048)
Glomus sp. 2	0.306 (0.029)	0.422 (0.065)
Acaulospora morrowiae	0.229 (0.025)	0.267 (0.055)
Entrophospora columbiana	0.259 (0.026)	0.385 (0.096)
Gigaspora margarita	0.239 (0.052)	0.326 (0.062)
Scutellospora heterogama	0.287 (0.029)	0.335 (0.042)

Shown are means, standard errors ($n = 5$; except for *E. columbiana* and *G. etunicatum*, $n = 4$), and statistical significance. First Dunnett's tests were used to compare each AMF inoculum effect with the control, and none of the pairs differed significantly ($P > 0.05$). We used Tukey–Kramer HSD to compare pairs only within the treatments that received AMF inocula (excluding control) and none of those were significantly different either ($P > 0.05$).

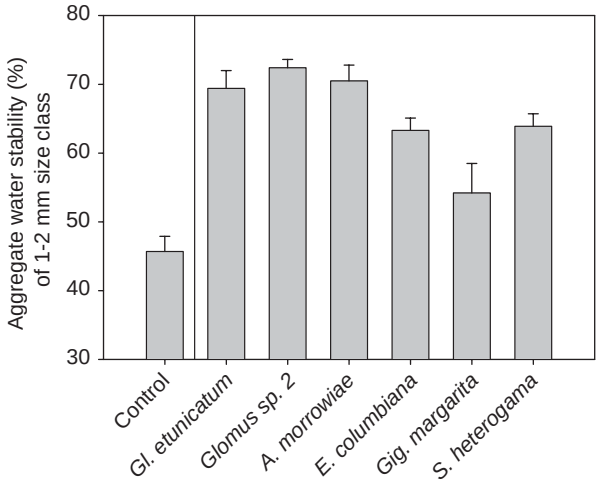

Figure. 2. Effect of AMF species inocula (without active AMF) on soil aggregate water stability (%) in the 1–2 mm size class. Control refers to pots with sterile AMF inoculum material added, but with *B. inermis* plants. Shown are means and standard errors of the mean ($n = 5$; except *E. columbiana*, $n = 4$). Accounting for differences in total PLFA amount in the inoculum, there was a significant difference in soil aggregation among different AMF species inocula (ANCVOA; $F_{AMF\ inoculum} = 5.44$; $P = 0.002$; test excludes the control).

differences in $WSA_{1-2\,mm}$ among the six different inoculation treatments ($F = 6.84$; $P = 0.0005$). To test if differences in $WSA_{1-2\,mm}$ could simply be explained by differences in microbial biomass among the different inocula, we used ANCOVA with the total amount of PLFA extracted from the microbial communities as the covariate. ANCOVA suggested that while this covariate explained a portion of the variability seen in $WSA_{1-2\,mm}$ ($F_{cov.\ PLFA} = 8.09$; $P = 0.009$), there was still a significant effect of different AMF species inocula ($F_{AMF\ inoculum} = 5.44$; $P = 0.002$).

Discussion

In this experiment we found differences in water-stability of aggregates when soils and roots were inoculated with inactivated AMF culture material of different AMF species originating from the same soil community. We provided three lines of evidence that live mycelium did not play a role in the observed differences: the lack of any AMF root colonization, the decrease in hyphal lengths compared to the control, and the decreased GRSP concentration in aggregates. We suggest that hyphal lengths and GRSP concentrations were decreased in the inoculated treatments due to decomposition by microbiota contained in the inoculum, but that more importantly, there was no new growth. Additionally, root biomass was not significantly different among the different inoculation treatments, making it highly unlikely that soil aggregation results were mediated by roots. The results can hence be best explained by the action of AMF-culture associated microbiota. It is important to emphasize that all AMF material used in this study was derived from the same soil using the same methods (including the same host plant), i.e. there is little potential that we made irrelevant comparisons among AMF-associated microbial communities from different ecosystems, which may have introduced artificially high variability among AMF isolates. Different geographical isolates of the same species of AMF have been shown to differ in a variety of characteristics, including association with soil bacterial communities (Andrade et al., 1997).

Differences in soil aggregation could have been caused by differences in the amount of organic material added to the pots as a function of the respective AMF inocula. This is highly improbable since (1) we used large amounts of soil (440 g per pot) with over 5% organic matter to which only a

small amount of the different inocula (20 g perlite) was added; (2) additionally, roots grew in all pots adding carbon through turnover and rhizodeposition. It is also unlikely that potential differences in nutrients among inocula played any role. The crushed and heated soils likely released large amount of nutrients (Johnson et al., 1995), compared to which inoculum could have contributed only a negligible amount; also, there were no differences in plant growth among the treatments.

The PLFA analysis revealed comparatively large differences among the microbial communities contained in the inocula of different AMF species (as evidenced by PCA), and we suggest that this difference was a driving force behind the patterns in $WSA_{1-2\,mm}$ we observed. Others have also found that microbes associated with AMF differ as a function of AMF species. Andrade et al. (1997) found significant differences in bacterial communities associated with different isolates and species of AMF grown with *Sorghum bicolor* L., even though all AMF used in this study were in the genus *Glomus*. The culture-dependent method used to characterize communities could have masked additional effects, and differences are likely to be greater among various genera of AMF (which differ in a number of biological/developmental traits; Hart and Reader, 2002), such as used in our study. Hence Andrade et al. (1997) likely underestimated the degree of potential variability among AMF species in their influence on associated microbiota. Importantly, bacteria have been isolated from individual AMF species, which are likely very important in soil aggregation, such as *Paenibacillus* spp. (Budi et al., 1999, Bezzate et al., 2000; Mansfeld-Giese et al., 2002; Hildebrandt et al., 2002) and others. Differential association with such bacteria among different AMF species could be a mechanism for the pattern we observed here, and future research should test whether some AMF isolates preferentially associate with specific bacteria (such as *Paenibacillus)* that have the propensity to be important in soil aggregation.

There is uncertainty regarding the correspondence of the microbial community in the pots after long-term greenhouse sub-culturing with the microbial community that these fungi would be associating with in situ. Innovative experimental and sampling designs are needed to address this question, and the question of how reproducible these microbial "enrichments" on hyphae of different AMF species can be. However, this problem is immaterial to the point we are making regarding the problems with interpreting ecological studies based on AMF pot culture material (not controlled for associated microbiota). At any rate, our data comparing biomass of bacteria in the inoculum between culture cycles (six culture cycles apart) suggest that these associations formed in the greenhouse are likely relatively stable.

The effect size we observed was rather large, soil aggregation increasing from 40% (in the planted control) to over 70% during this short-term study. Our study was designed to maximize effect sizes, since we broke up macro-aggregates prior to the inoculation with AMF-associated microbiota, creating a population of non-stable aggregates in the 1–2 mm size class. Macro-aggregates did not necessarily have to be formed de novo by hierarchically arranging micro-aggregates with various binding agents into macro-aggregates (Six et al., 2000) during the experiment. Rather, existing, but unstable aggregates could have been stabilized by more simple "mending" processes. Nevertheless, incomplete disaggregation is probably quite common in soils in response to disturbance, so this may not be an unrealistic scenario.

Our results highlight that a more mechanistic understanding of AMF contributions to soil aggregation (or other ecosystem processes) can only be derived by separating out effects of the live mycelium from those of microbiota accompanying the symbiosis. Future studies should be aimed at dissecting the communities of microbiota associated with different AMF species in greater detail. It is also important to test alternative experimental designs to the one used here, e.g. using extraction of microbiota rather than AMF inactivation and, conversely, inactivating prokaryotes contained in the AMF inoculum to compare effects of live AMF with and without associated microbiota. From our experiment, it is unclear whether the microbiota important in the response we observed were associated with the initially alive fungal mycelium (hyphae and spores), with the decomposing mycelium (after it was killed in the experiment or as a consequence of normal mycelium turnover), the roots of the AMF host, and/or the growth substrate itself. All of these microbial communities were added in the experimental design we used here. Subsequent studies using fractionation of these different compartments (roots, rhizosphere, AMF mycelium) may be used to further differentiate effects.

Although previous studies that have used various single species cultures to test effects of diversity of AMF on ecosystem processes (e.g. van der Heijden et al., 1998) may have to be re-interpreted in light of our results, the differences in soil aggregation we observed were still an AMF species effect (albeit not one mediated directly by live mycelium). A weakness of the present experimental design is

that we cannot ascertain effect partitioning among AMF mycelia and associated microbes; while we have shown that culture-associated microbes can potentially be important in soil aggregation, future research needs to address their *relative* significance in the presence of AMF mycelia and/or roots.

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