Phylogeny of arbuscular mycorrhizal fungi predicts community composition of symbiosis-associated bacteria

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Abstract

Many physicochemical and biotic aspects of the soil environment determine the community composition of bacteria. In this study, we examined the effects of arbuscular mycorrhizal fungi, common symbionts of higher plants, on the composition of bacterial communities after long-term (7–8 years) enrichment culture in the presence of a plant host. We showed that the phylogeny of arbuscular mycorrhizal fungal isolates was a highly significant predictor of bacterial community composition, as assessed by cluster analysis, redundancy analysis and linear discriminant analysis of phospholipid fatty acid patterns. Numerous phospholipid fatty acids differed between the phylogenetic groupings; this pattern also held for fungal-origin phospholipid fatty acids and in a combined bacterial/fungal analysis, suggesting that categorizing phospholipid fatty acids into predominantly bacterial and fungal origin did not affect the overall outcome. The mechanisms underlying this observation could include substrate quality (and quantity) effects, interactions mediated by the host plant (e.g. rhizodeposition) and direct biotic interactions between arbuscular mycorrhizal fungi and bacterial populations. Our results suggest that aspects of arbuscular mycorrhizal fungal functions may be partially explained by the symbiosis-accompanying bacterial communities, a possibility that should be explicitly considered in studies examining the roles of arbuscular mycorrhizal fungal species diversity in soil and ecosystem processes.

Introduction

The composition of soil biotic communities is determined by a number of factors, including the physicochemical attributes of the substrate in space and time, and biotic interactions within and between trophic levels (e.g. predation/ingestion, competition, facilitation). In this paper, we explore a new predictor of community composition, the phylogeny of a trophic level below that for which community composition is to be explained (Eom *et al.*, 2000; Wimp *et al.*, 2004). As a model system, we used arbuscular mycorrhizae and bacterial communities that derive their carbon from these root–fungal symbioses. We chose to focus on arbuscular mycorrhizal symbioses as the vast majority of terrestrial plants form associations with arbuscular mycorrhizal fungi (Smith & Read, 1997), and bacterial communities that inhabit the rhizosphere are dependent on mycorrhizae (either the fungi, root or both) for their carbon resources.

Arbuscular mycorrhizal fungi that associate with plant roots can differ significantly in life history traits, such as speed of growth and investment in internal and external structures (Hart & Reader, 2002). As a consequence of these variations, they can also differ functionally with respect to effects on the host (e.g. Klironomos, 2003; Munkvold *et al.*, 2004). Given these differences, it is perhaps not surprising that arbuscular mycorrhizal fungi can also differentially influence soil bacterial communities associated with the symbiosis (e.g. Andrade *et al.*, 1997). Although numerous studies have documented changes in the mycorrhizosphere bacterial community in response to the presence and/or identity of arbuscular mycorrhizal fungi (using a variety of community fingerprinting methods; reviewed in Hodge, 2000; Johansson *et al.*, 2004), to date it has not been possible to relate changes in bacterial composition to changes in the composition of the arbuscular mycorrhizal symbiosis.

A possible predictor may be the phylogeny of the arbuscular mycorrhizal fungal partner in the symbiosis.

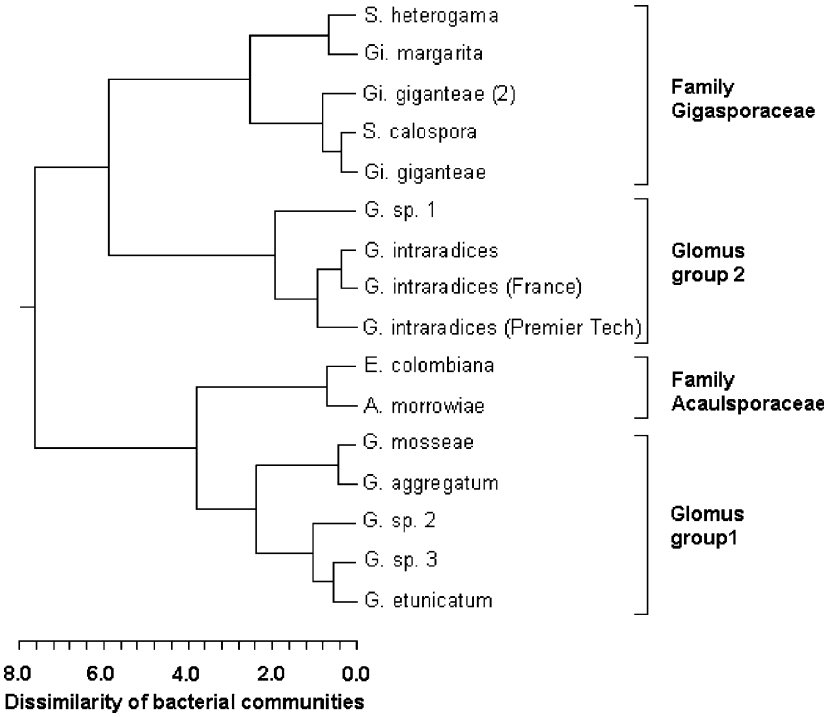

Fig. 1. Euclidean distance dendrogram generated using Ward's method for soil bacterial communities [based on phospholipid fatty acid (PLFA) analysis]. Abbreviations for arbuscular mycorrhizal fungal genus names are as follows: Gi, *Gigaspora*; Gl, *Glomus*; E, *Entrophospora*; A, *Acaulospora*; S, *Scutellospora*. Cluster analysis indicates the separation of bacterial communities according to four arbuscular mycorrhizal fungal phylogenetic groups: *Glomus* group 1, *Glomus* group 2, Gigasporaceae (group 3) and Acaulosporaceae (group 4).

Arbuscular mycorrhizal fungal phylogeny is a useful predictor of many characteristics of fungal growth (Hart & Reader, 2002). It is possible that such phylogeny effects can propagate through the various levels of biological hierarchy (genetics, biochemistry, substrate quality) and contribute to differences in bacterial community composition.

We tested for effects of arbuscular mycorrhizal fungal clade identity on symbiosis-associated bacterial communities using arbuscular mycorrhizal fungal species cultured from the same ecosystem to avoid confounding factors of introducing different bacterial sources. For example, different geographical isolates of the same species of arbuscular mycorrhizal fungus have been shown to differ in a variety of characteristics, including association with soil bacterial communities (Andrade *et al.*, 1997). Our approach was to use long-term single-species cultures of arbuscular mycorrhizal fungi, essentially having 'enriched' or selected for bacterial communities associating with the symbiosis.

Materials and methods

Arbuscular mycorrhizal fungal single-species cultures and bacterial 'enrichment'

We used a long-term (7–8 years) enrichment culture approach, for which isolates of different arbuscular mycorrhizal fungal species were obtained from an old field ecosystem and cultured on a common host plant species. Arbuscular mycorrhizal fungi originated from the Long-Term Mycorrhiza Research Site (LTMRS; Klironomos, 2003) at the University of Guelph (Guelph, ON, Canada). The isolates represented several clades of arbuscular mycorrhizal fungi (listed in Fig. 1). In addition to isolates from the LTMRS, we also included two isolates of *Glomus intraradices* from elsewhere (France and an industrial isolate) to explore potential effects related to the geographical origin of the isolates. These cultures were grown for 7–8 years in a glasshouse under standardized conditions (Klironomos, 2003), all passing through rotations of sterilized (autoclaving, twice for 1 h) soil, perlite or turface media with leek (*Allium porrum*; two plants per pot) as the host plant. The pots were watered once a week with 500 mL of water, or as needed, and fertilized every 2 weeks with 300 mL of a Long-Ashton Nutrient solution (low in phosphorus; Klironomos, 2003). For each culture cycle, one-quarter of the contents (including other microbiota, roots and arbuscular mycorrhizal fungal 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 arbuscular mycorrhizal fungi were grown in perlite substrate. After 5 months, the cultures were harvested, dried, the roots were chopped into small fragments, and the pot contents were placed in plastic bags for use in this study. Root and shoot dry biomass of leek plants (on a per pot basis; $n = 8$) was also recorded, and arbuscular mycorrhizal fungal hyphal lengths (expressed as m per g perlite substrate; $n = 8$) were measured as described in Hart & Reader (2002).

Phospholipid fatty acid (PLFA) analysis

Arbuscular mycorrhizal fungal substrate (perlite) was analyzed for bacterial community structure using PLFA analysis. PLFAs were extracted (from two replicate 30 g samples of inoculum for each species/isolate; these values were averaged before conducting statistical analyses), and analyzed according to White & Ringelberg (1998). Briefly, lipids were removed from samples into chloroform using a modified Bligh & 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, coelution with purchased standards and comparison of samples between capillary columns of differing polarity [HP-5 (cross-linked 5% phenyl methyl silicone), 50 m × 0.32 mm × 0.52 μm film; HP-225 (50% cyanopropylphenyl methylpolysiloxane), 30 m × 0.32 mm × 0.25 μm film; Hewlett-Packard, Palo Alto, CA]. Final verification of peak identity was made by gas chromatography/mass spectroscopy on representative samples. FAMEs were quantified on an HP-225 column using an HP 6890 series gas chromatography system (Hewlett-Packard) and a protocol according to Frostegård *et al.* (1993). PLFAs were summed per sample to obtain the total amount (nmol PLFA per g inoculum) as an indication of total microbial biomass. Arbuscular mycorrhizal fungi themselves produce a variety of fatty acids (Graham *et al.*, 1995; Bentivenga & Morton, 1996). In order to avoid confounding effects, we analyzed PLFAs of predominantly fungal and bacterial origin separately (Frostegård & Bååth, 1996), as listed in Table 1.

Table 1. Mean relative proportions of phospholipid fatty acids (PLFAs) by arbuscular mycorrhizal group

	Group			
PLFA	1	2	3	4
Bacterial markers				
15:0	2.76a	3.33b	2.77a	4.10c
17:0	4.48a	2.93a	6.56b	2.53a
u14:1	1.11	1.02	0.86	1.27
i15:1	2.09	0.92	1.17	1.98
i15:0	10.79	12.05	12.29	8.68
i16:1	1.45a	1.23a	1.99a	3.42b
i16:0	5.86a	13.04b	8.18c	6.72ac
i17:0	5.87a	5.57a	7.02b	5.89ab
a15:0	7.81a	3.31b	3.99b	6.87a
a17:0	3.41a	2.28b	3.11a	3.47a
br17:0	3.82a	2.91b	4.81c	4.19a
16:ω9	2.11a	2.16a	3.34b	1.68a
17:1ω8c	4.64a	6.19a	4.97a	9.71b
cy17:0	7.34a	7.28	6.83	7.24
cy19:0	22.74a	18.16b	15.26c	19.87bd
10me16:0	8.06a	10.26b	12.53c	5.17d
10me17:0	1.17a	0.36b	0.80ab	2.65c
10me18:0	4.49a	6.98b	3.49a	4.52a
Fungal/AMF markers				
14:0	2.28a	1.24ab	0.72b	2.87a
16:1ω7c	5.80a	5.97a	5.67a	4.64a
16:1ω5	2.72a	1.20b	2.13c	2.40ac
16:0	18.10a	12.21b	19.13a	18.85a
18:3	0.65	0.20a	0.14a	1.00
18:2ω6	0.89a	0.40b	0.64ab	0.96a
u18:1	3.30a	3.49ab	4.66b	3.22ab
18:1ω9	6.33a	7.25a	6.27a	6.80a
18:1ω7	8.21a	8.64a	10.44	8.55a
18:0	4.83a	3.91a	7.06	4.38a

The groups are (see also Fig. 1): *Glomus* group 1, *Glomus* group 2, Gigasporaceae (group 3) and Acaulosporaceae (group 4).
Groups followed by the same letter are not significant at $\alpha = 0.05$.
AMF, arbuscular mycorrhizal fungi.

Statistics

We performed statistical analyses on the mole-percentages of different PLFA species to capture the overall microbial community differences between samples, using only the 18 most common bacterial PLFAs that were present in all samples (additional fungi-derived PLFAs; Table 1). Importantly, this procedure avoided the inclusion of zero mole-percentages, which could lead to biased comparisons that are possible when using high and low biomass samples. We conducted all analyses using the mean PLFA profiles of two replicates per arbuscular mycorrhizal fungal isolate, thus avoiding pseudoreplication. Initial data exploration consisted of cluster analysis (Ward's method, Euclidean distance).

Relationships between the PLFA data of all samples were examined using ordination techniques. Initial detrended correspondence analysis indicated that the data exhibited a linear, rather than unimodal, response to sample origin, justifying the use of linear ordination methods (Lepš & Šmilauer, 2003). Therefore, relationships between sample groups suggested by cluster analysis were evaluated by redundancy analysis using CANOCO software (Microcomputer Power, Ithaca, NY). This technique finds the portion of the variability that can be explained by experimental treatments. A Monte Carlo permutation test based on 499 random permutations was used to test the null hypothesis that bacterial community profiles were unrelated to arbuscular mycorrhizal fungus identity.

The classification of potential data groupings, suggested by cluster analysis, was also conducted using discriminant analysis. Discriminant analysis is used to classify different arbuscular mycorrhizal fungal species in categories based on a series of PLFA biomarkers. The correct rate of classification can be used to evaluate the relationships between categories. The rate of correct classification was calculated by self-crossing the database, and is the percentage of cases that are classified by the analysis in the correct category.

Differences in hyphal lengths, root biomass and shoot biomass, and between individual PLFAs of the different arbuscular mycorrhizal fungal groups (according to Schüssler *et al.*, 2001), were analyzed by one-way analysis of variance (ANOVA) using the SPSS for Windows software package (version 13) (SPSS Inc., Chicago, IL). Replication was $n = 8$ per arbuscular mycorrhizal fungal species/isolate; hence, the following levels of replication were given for the different groups: *Glomus* group 1 (five isolates, $n = 40$); *Glomus* group 2 (four isolates, $n = 32$); Gigasporaceae group 3 (five isolates, $n = 40$); Acaulosporaceae group 4 (two isolates, $n = 16$).

Results

Cluster analysis suggested the presence of four distinct groups of bacterial communities diverging in association with arbuscular mycorrhizal fungal species or clades (Fig. 1). Specifically, bacterial communities clustered corresponding to arbuscular mycorrhizal fungal groups as follows: (1) *Glomus* species group 1; (2) *Glomus* species group 2 (comprising mainly *Glomus intradices* isolates); (3) family Gigasporaceae (comprising genera *Gigaspora* and *Scutellospora*); (4) family Acaulosporaceae (comprising *Acaulospora* and *Entrophospora* species) (Fig. 1).

Redundancy analysis was used to further assess whether bacterial communities corresponded to arbuscular mycorrhizal fungal groupings. Redundancy analysis results were displayed on triplots depicting the relationship between arbuscular mycorrhizal fungal groups and individual PLFAs (Fig. 2a). We also plotted PLFAs of predominantly fungal origin separately (Fig. 2b), revealing a clear separation of the groups. However, as we were mostly interested in bacterial communities (and as arbuscular mycorrhizal fungi would obviously also contribute to the fungal-derived PLFAs), we restricted the remaining analyses to bacterial PLFAs. Their relationship with hyphal length, root and shoot biomass is presented in Fig. 3. The first and second canonical axes explained 34.8% and 13% of the variability in the dataset, respectively. A Monte Carlo permutation test indicated that the variability accounted for by the first axis was significant (F ratio = 6.42; $P = 0.002$). Testing all canonical axes together confirmed that bacterial community composition was significantly affected by arbuscular mycorrhizal fungus group identity (trace = 0.487; F ratio = 3.79; $P = 0.002$). Comparison of individual PLFAs indicated that a number were significantly different between groups (Table 1).

A linear discriminant analysis with cross-validation was also used to classify the four groups. Wilks Λ for the model was less than 0.001 ($P < 0.001$), and the expected groupings were classified 100% correctly.

Significant differences were found between the hyphal lengths of all groups, with the exception of the Acaulosporaceae, whose hyphal length was not significantly different from that of *Glomus* groups 1 and 2 (Fig. 4). The arbuscular mycorrhizal fungal 'marker' PLFA 16:1ω5 was negatively correlated ($r = -0.48$) with hyphal lengths across the fungal groups ($n = 4$). The hyphal lengths of the Gigasporaceae were significantly greater than those of the other groups. Conversely, Gigasporaceae-infected plants exhibited significantly reduced root and shoot biomass, whereas no significant differences were found for this measure between plants infected with other arbuscular mycorrhizal fungal inoculum groups (Fig. 4).

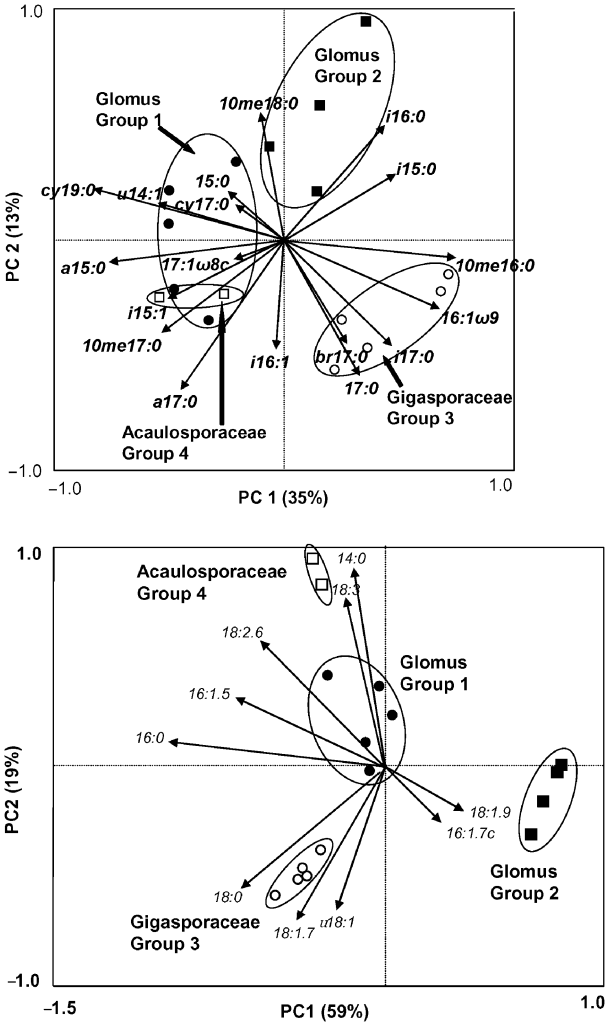

Fig. 2. Redundancy analysis of phospholipid fatty acid (PLFA) variables for each arbuscular mycorrhizal fungal group. Vectors (arrows) indicate correlations between each PLFA and each sample. PLFA vectors pointing towards a sample indicate a positive correlation, whereas vectors pointing away indicate a negative relationship (longer arrows indicate stronger relationships). Individual data points are means ($n = 2$) for each isolate/species. Analyses are presented for PLFAs of predominantly bacterial (a) and fungal (b) origin, with PLFAs as given in Table 1.

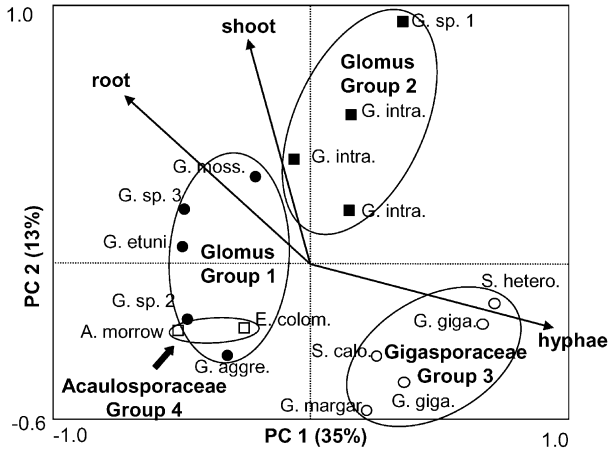

Fig. 3. Redundancy analysis of phospholipid fatty acid (PLFA) variables for each arbuscular mycorrhizal fungal group. Vectors for hyphal length and root and shoot biomass are superimposed, indicating their relative correlation with arbuscular mycorrhizal fungal group. Individual data points are means ($n = 2$) for each isolate/species.

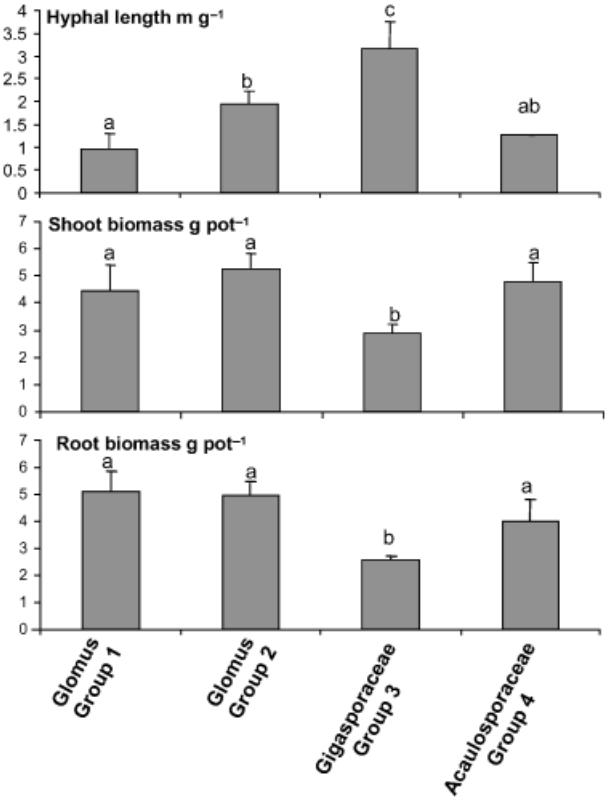

Fig. 4. Hyphal lengths of arbuscular mycorrhizal fungi in the perlite substrate, and root and shoot biomass of the host plant (leek) grown with representatives of the four different arbuscular mycorrhizal clades (error bars are standard errors of the mean; see 'Materials and methods' for replication levels).

Discussion

Biotic interactions are known to be important in regulating community composition. Yet, only a few studies (Eom *et al.*, 2000; Wimp *et al.*, 2004) have shown that the phylogeny of a trophic level (here arbuscular mycorrhizal) can influence the community composition of a higher trophic level (here soil bacteria). The arbuscular mycorrhizal fungus material used here was obtained from the same soil using the same methods (including the same host plant), i.e. there was little potential to make irrelevant comparisons between arbuscular mycorrhizal fungus-associated microbial communities from different ecosystems, which may have introduced artificially high variability amongst arbuscular mycorrhizal fungal isolates. Different geographical isolates of the same species of arbuscular mycorrhizal fungus may differ with regard to a variety of characteristics, including association with bacterial communities (Andrade *et al.*, 1997). However, this appeared not to be the case in our study: the communities associated with the different *Glomus intraradices* isolates all clustered together. This suggests that the results may be generalizable beyond the particular isolates used here.

Bacterial community composition

Redundancy analysis triplots indicated a distinct separation of samples on the basis of arbuscular mycorrhizal fungal inoculation group (Figs 2a and 3). The arbuscular mycorrhizal fungal genus *Glomus* is polyphyletic (Schüssler *et al.*, 2001); hence, the separation into two groups is unsurprising. The closer the alignment of the vector for an individual variable to an ordination axis, the more that variable can be used to explain data variation along that axis. From Fig. 2a and Table 1, it can be seen that a number of individual PLFAs clearly drive the separation of each group. The use of PLFAs to represent specific populations of microorganisms can be problematic as a result of overlap in the PLFA composition of organisms, our lack of knowledge regarding the PLFA composition of a soil organism, and conformational shifts in membrane PLFAs in response to stress and nutritional status (Guckert *et al.*, 1986; Zelles *et al.*, 1994). In spite of these difficulties, PLFA data are commonly used to make inferences about the microbial community composition of soil samples. In our dataset, the fatty acids i17:0 and br17:0, which are commonly used as markers for Gram-positive bacteria (Bossio & Scow, 1998; Griffiths *et al.*, 1999; Pankhurst *et al.*, 2001), were significantly greater in group 3 samples (Table 1) and important to the separation of this group from others (Fig. 2). However, with this exception, it is difficult to make inferences regarding selection for specific bacterial groups. For example, although fatty acids 10me16:0, 10me17:0 and 10me18:0 are thought to be mainly produced by actinomycetes (Kroppenstedt, 1985; Zelles

et al., 1994), vectors for each were dissimilarly aligned (Fig. 2). Similarly, fatty acids which are commonly associated with Gram-positive bacteria (e.g. a15:0, i16:0 and i17:0) were not uniformly more abundant in any one group (Table 1). These results suggest that methods which afford greater resolution than those employed in this study will be necessary to fully elucidate the changes in bacterial community composition driven by arbuscular mycorrhizal fungal species composition.

Fungal-derived PLFAs (Fig. 2b, Table 1) also separated clearly into four groups. This may be partially reflective of differences in associated microfungal communities, as hyphae not belonging to arbuscular mycorrhizal fungi were observed microscopically in the preparations (M. C. Rillig, unpublished observation). However, these PLFAs cannot be easily separated from those derived from arbuscular mycorrhizal fungi, and hence no firm conclusions can be drawn. Another tempting conclusion is that profiles of PLFAs may be useful to separate coarse-level arbuscular mycorrhizal fungal groupings, at least under artificial conditions (substrate), which might be worthy of further study (Graham *et al.*, 1995; Bentivenga & Morton, 1996).

Potential mechanisms

The mechanisms underlying our observed pattern cannot be clearly discerned from this study, but several possibilities for microbial community divergence exist. Altered bacterial communities may be a direct consequence of either the tissue quality and/or quantity of arbuscular mycorrhizal fungal hyphae and spores (Figs 3 and 4). Examples of different biochemical quality of arbuscular mycorrhizal fungal mycelia from different species include differential content of the recalcitrant proteinaceous substance glomalin (Wright & Upadhyaya, 1998) or of fatty acids (Graham *et al.*, 1995). In addition to direct nutritional effects, direct biotic interaction of arbuscular mycorrhizal fungal mycelia and bacterial populations, for example through fungal production of stimulatory or inhibitory compounds, may also have contributed to the observed effect. For example, a direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and other rhizosphere microbes has been documented under controlled *in vitro* conditions (Filion *et al.*, 1999). Finally, it is important to emphasize that our enrichment culture, owing to the obligate biotrophic nature of arbuscular mycorrhizal fungi, also included the plant host, not just the arbuscular mycorrhizal fungus. This suggests that the arbuscular mycorrhizal fungal isolates may also have influenced bacterial communities via their effects on the host (e.g. on rhizodeposition) or root growth (Figs 3 and 4). The latter was illustrated in an experiment employing a split-root design, where direct mycorrhizal effects of two arbuscular mycorrhizal fungi colonizing maize roots could be separated from those of plant-mediated effects on bacterial communities (Marschner & Baumann, 2003).

Consequence for mycorrhizal biology

In a previous glasshouse study, using a subset of the same arbuscular mycorrhizal fungi cultures as studied here, we were able to provide evidence suggesting that symbiosis-cultured bacterial communities could be functionally different (Rillig *et al.*, 2005). In particular, we suggested that the microbial communities could differentially influence soil aggregation, an important ecosystem process long known to be affected by arbuscular mycorrhizal fungi (Tisdall & Oades, 1982; Rillig, 2004). It still remains to be tested what is the relative contribution of arbuscular mycorrhizal fungi and the symbiosis-associated microbial communities to this or any other process. However, it is an intriguing possibility that associated microbial communities, inevitably contained in the inoculum of arbuscular mycorrhizal fungal single-species cultures (except for the few isolates available as *in vitro* material), could be contributing to processes hitherto ascribed to the arbuscular mycorrhizal fungi themselves. The present study indicates that this potential influence may occur in a fashion consistent with arbuscular mycorrhizal fungal clade affiliation, and hence fungal life history strategy (Hart & Reader, 2002). This is particularly interesting as life history strategy has also been used as a predictor of arbuscular mycorrhizal fungal functionality (Hart & Reader, 2002).

Conclusions

There is uncertainty regarding the correspondence between the microbial community in our pots after long-term glasshouse subculturing and the microbial community that these fungi would be associated with in the field. Nevertheless, it is these pot-borne microbial communities that would also be present in many mycorrhizal ecology studies. Our study used PLFA as a fingerprinting tool; we chose PLFA as it has been shown to be more sensitive than DNA-based methods (Dierksen *et al.*, 2002), but it has the disadvantage of a lower resolution. Hence, future work should also include DNA-based community analysis methods. Furthermore, even though our study was restricted to the microbial level, it is tempting to speculate that other soil biota groups, such as nematode (Bakhtiar *et al.*, 2001) and microarthropod (Gange, 2000) communities, may also experience a similar 'selection' based on arbuscular mycorrhizal fungal clade identity, for example based on differential palatability or nutritional quality of arbuscular mycorrhizal fungi.

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