Relationship between assemblages of mycorrhizal fungi and bacteria on grass roots

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Summary
Soils support an enormous microbial diversity, but the ecological drivers of this diversity are poorly understood. Interactions between the roots of individual grass species and the arbuscular mycorrhizal (AM) fungi and bacteria in their rhizoplane were studied in a grazed, unimproved upland pasture. Individual root fragments were isolated from soil cores, DNA extracted and used to identify plant species and assess rhizoplane bacterial and AM fungal assemblages, by amplifying part of the small-subunit ribosomal RNA gene, followed by terminal restriction fragment length polymorphism analysis. For the first time we showed that AM fungal and bacterial assemblages are related in situ and that this relationship occurred at the community level. Principal coordinate analyses of the data show that the AM fungi were a major factor determining the bacterial assemblage on grass roots. We also report a strong influence of the composition of the plant community on AM fungal assemblage. The bacterial assemblage was also influenced by soil pH and was spatially structured, whereas AM fungi were influenced neither by the bacteria nor by soil pH. Our study shows that linkages between plant roots and their microbial communities exist in a complex web of interactions that act at individual and at community levels, with AM fungi influencing the bacterial assemblage, but not the other way round.

Introduction
Soils contain an extraordinary range of microorganisms, their diversity being several orders of magnitude greater than the communities found in aquatic systems (Torsvik et al., 2002; Gans et al., 2005). Pasture soils are no exception and have been estimated to contain up to 1 million species g⁻¹ (Curtis et al., 2006). However, despite the ecological importance and vast diversity of soil microorganisms, past practical and theoretical constraints have limited our ability to document patterns of soil microbial diversity and understand the factors that regulate their community assemblage (Homer-Devine et al., 2004).

Primary productivity is believed to be a key determinant of soil microbial diversity (Kaunzinger and Morin, 1998; Homer-Devine et al., 2003). However, the ecological mechanisms by which microbial communities interact with plant species are poorly understood, with several studies showing rhizosphere microbial assemblages to be influenced by plant species (Miethling et al., 2000; Smalla et al., 2001; Kowalchuk et al., 2002), soil type (Buckley and Schmidt, 2003; Girvan et al., 2003; de Ridder-Duine et al., 2005) or a combination of both factors (Alvey et al., 2003; Singh et al., 2006). In grazed pastures, the spatial variations in bacterial assemblages can be influenced largely by the soil nutrient status, which in turn varies due to the presence of historical urine patches affecting soil pH (Ritz et al., 2004).

Most studies of the interactions between plants and soil microbial assemblage have investigated rhizosphere soils (defined as those influenced by plant roots). Plants provide carbon to rhizosphere microorganisms in the form of rhizodeposits, which, despite being a small proportion of the total C inputs from vegetation to soil, are believed to select for a beneficial microbial community in the rhizosphere (Jones et al., 2004; Singh et al., 2004). However, the specific effects of plants are likely to be greatest on the rhizoplane, where an active microbial assemblage will utilize rhizodeposits, before diffusion of the residue into the rhizosphere soil.
Studying the rhizoplane microbial community assemblage in field and in a mixed plant community is difficult, because roots from different plants are intermingled and difficult to identify. Extraction of DNA from the root fragments for identification of both plant species and analysis of the soil (rhizoplane) microbial assemblages has been used (Ridgway et al., 2003), but failed to show a relationship between bacterial assemblage and plant species although an influence of plant community composition was found (Nunan et al., 2005). Fungi can also be important in shaping the soil bacterial assemblages. Artursson and colleagues (2006) reviewed interactions between arbuscular mycorrhizal (AM) fungi and soil bacteria and, in a pot experiment, demonstrated selection of distinct bacterial populations following inoculation of the AM fungus Glomus mosseae in soils (Artursson et al., 2005). Furthermore, studies on multidirectional interactions among vegetation, fungi and bacteria at community levels in field conditions have not been reported. The aim of the present study was to determine interactions between plants and the bacterial and AM fungal assemblages (which typically colonize grass species) in the rhizoplane of grass roots in a grazed, upland pasture. We tested the hypotheses that: (i) a multidirectional interaction exists between plant community, AM fungi and rhizoplane bacterial assemblage; (ii) this interaction is facilitated at the community level; and (iii) there was a significant relationship between mycorrhizal and rhizoplane bacterial assemblages. We also examined the effect of soil pH on bacterial and fungal assemblages and its impact on relationship between these two assemblages.

Results and discussion

Interactions between plants, AM fungi and rhizoplane bacterial assemblages

Forty regularly spaced, spatially referenced cores were sampled. The relative abundance of the main plant species present in the cores and the soil pH are shown in Table 1. A total of 314 root fragments from the cores were used for plant identification and microbial community analysis. Each root fragment was identified to species level by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of the plastid leucine transfer RNA (trnL, UAA) gene intron (Ridgway et al., 2003). The AM fungal and bacterial assemblages on each root fragment were determined by amplifying a fragment of the small-subunit ribosomal RNA gene (SSU rRNA) using the (PCR), followed by terminal restriction fragment length polymorphism (TRFLP) analysis (Vandenkourhuyse et al., 2002; Nunan et al., 2005). The variability in the sequence of target SSU rRNA genes (18S and 16S rRNA genes for fungi and bacteria, respectively) was used to sort samples into operational taxonomic units (OTUs). Members of different species may belong to the same OTU, as TRFLP operates at a resolution other than species. However, it is a consistent measure of community composition and a difference between two OTUs sets directly reflects a difference in species composition (Singh et al., 2004; 2006). The number of OTUs obtained for AM fungi and bacterial assemblages sampled with roots of each plant species are presented in Table 2. Between 6.8 and 9.28 fungal OTUs, and between 32 and 41.6 bacterial OTUs, were obtained from different plant roots. Statistical analysis using residual maximum likelihood (REML) showed that there was no significant difference in the number of bacterial or fungal OTUs obtained from different plants. Principal coordinate (PCO) analyses confirmed that the composition of AM fungal, but not that of bacterial assemblages, was influenced by plant root species. The similarity of assemblages associated with root fragments irrespective of plant composition from within the same core was, however, greater than that of assemblages from different cores for both AM fungal and bacterial assemblages (Fig. 1). This suggests that there was a ‘local environment’ effect due to either soil characteristics within the

### Table 1. The range of soil pH and plant species abundance (percentage) present in the 40 sample cores.

<table>
<thead>
<tr>
<th>Core attribute</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil pH</td>
<td>4.5 ± 0.26</td>
<td>3.97–5.21</td>
</tr>
<tr>
<td>Plant species (% abundance)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agrostis capillaris</td>
<td>60 ± 23.9</td>
<td>0–89</td>
</tr>
<tr>
<td>Agrostis vinealis</td>
<td>12 ± 15.8</td>
<td>0–54</td>
</tr>
<tr>
<td>Festuca juncifolia</td>
<td>2 ± 4.3</td>
<td>0–15</td>
</tr>
<tr>
<td>Festuca rubra</td>
<td>7 ± 11.1</td>
<td>0–42</td>
</tr>
<tr>
<td>Poa pratensis</td>
<td>5 ± 6.7</td>
<td>0–23</td>
</tr>
<tr>
<td>Deschampsia cespitosa</td>
<td>8 ± 16</td>
<td>0–57</td>
</tr>
<tr>
<td>Other species</td>
<td>5 ± 8.8</td>
<td>0–33</td>
</tr>
</tbody>
</table>

Most of the other species belong to Holcus spp. or Calamagrostis spp.

### Table 2. Average number of OTUs associated with plant species.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Bacterial OTUs</th>
<th>Fungal OTUs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrostis capillaris</td>
<td>35.65</td>
<td>8.44</td>
</tr>
<tr>
<td>Agrostis vinealis</td>
<td>33.24</td>
<td>9.29</td>
</tr>
<tr>
<td>Holcus spp.</td>
<td>32.00</td>
<td>8.50</td>
</tr>
<tr>
<td>Festuca juncifolia</td>
<td>35.83</td>
<td>6.88</td>
</tr>
<tr>
<td>Festuca rubra</td>
<td>35.44</td>
<td>7.80</td>
</tr>
<tr>
<td>Poa pratensis</td>
<td>32.46</td>
<td>7.08</td>
</tr>
<tr>
<td>Deschampsia cespitosa</td>
<td>37.72</td>
<td>7.70</td>
</tr>
<tr>
<td>Calamagrostis spp.</td>
<td>41.60</td>
<td>8.09</td>
</tr>
</tbody>
</table>

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The relationship between different assemblages in samples and intersample separation distance was quantified by using semivariograms. The semivariogram measures the spatial variation of a phenomenon by describing how sample data are related as a function of distance and direction. In general, two closely neighbouring points are more likely to have similar values than two points farther apart (Ritz et al., 2004; Nunan et al., 2005). Semivariance analysis of PCO scores revealed that AM fungal assemblage was not spatially structured at the inter-core scale (> 2 m) (Fig. 2A), nor was it influenced by the soil pH. However, assemblages associated with root fragments from within a core were more similar than those from different cores. This suggested that the composition of vegetation surrounding a root fragment influenced the composition of AM fungi associated with that fragment (Fig. 2B). Thus, the AM fungal assemblage was influenced both by the composition of the plant community and by individual plant species colonized.

The effect of the plant community on both bacterial and AM fungal assemblages was further tested by Procrustean analysis. We chose Procrustean analysis...
over the more commonly used Mantel test because it is more powerful for detecting matrix associations, particularly when the number of observations is high (Peres-Neto and Jackson, 2001) as is the case here. There was significant concordance between AM fungal and plant communities ($m_{12} = 0.28; P < 0.001$), suggesting that plant community composition had an important influence on fungal assemblages associated with individual plants. In order to determine whether the relationship between AM fungal assemblages and plant communities was influenced by pH or individual plant identity, the Procrustes residuals were analysed, but no relationship was found (data not shown). This means that the relationship between plant community and AM fungal assemblage was influenced neither by the pH of the local environment, nor by the identity of the plant to which the microbial communities were associated. Previous studies have shown that roots of several plant species are colonized by unique mycorrhizal communities (Bidartondo et al., 2002; Vandenkoornhuyse et al., 2002; 2003). In grassland ecosystems, most of the plants are perennial and obligate biotrophs such as AM fungi compete for the same niche, the plant root. It has been suggested that competition among AM fungi for a limited carbon source drives the preference of the partners in AM symbiosis (Vandenkoornhuyse et al., 2003). However, the observation that the surrounding plant community influences AM fungal diversity of an individual plant species is novel and supports Wilkinson’s hypothesis (1997) that plants which reproduce vegetatively or have short-distance seed dispersal, come into contact with genetically identical fungi. This corresponds to a ‘pseudo-vertical transmission’ that makes mutualism likely to evolve. In such a model, the AM fungi would become adapted both to the host plants and to the immediately surrounding environment.

The composition of bacterial assemblages associated with root fragments of different plant species was not significantly different. Spatial structure was found for the first two PCO dimensions (Fig. 3A), which in turn were significantly correlated with soil pH ($P < 0.001$). The semivariance of bacterial PCO scores in the third dimension increased as a function of vegetation profile distance, indicating that samples from cores with a similar vegetation composition, regardless of their respective location within the plot, were more similar than the samples from cores with dissimilar vegetation. (Fig. 3B). The Procrustes analysis showed a significant concordance between assemblage of bacteria and plant community ($m_{12} = 0.31; P < 0.001$). Therefore, despite the lack of an effect of individual plant species, the composition of plant community within a core contributed to a local environment that influenced the bacterial assemblages.

![Fig. 3. Rhizoplane bacterial community structure is affected by spatial location, vegetation composition and AM fungal community structure.](image)

**Fig. 3.** Rhizoplane bacterial community structure is affected by spatial location, vegetation composition and AM fungal community structure. The semivariance for the PCO dimensions between sample pairs was calculated as a function of euclidean distance or vegetation distance (distance = 1 – composition similarity). The figures show semivariance as a function of (A) separation distance for PCO scores in the second dimension and (B) as a function of vegetation distance in the third dimension of bacterial community data (○), and when sample pairs were grouped according to AM fungal similarity, mean AM fungal similarities were 0.95 (■), 0.8 (▲), 0.6 (x) and < 0.5 (▲). Dashed lines are bootstrap 95% confidence intervals obtained from 1000 random samplings of measured data. Semivariance values of sample pairs containing highly similar AM fungal communities (■) were lower than the values of sample pairs with less similar communities, at all separation distances up to 6 m and at all vegetation distances.

**Interactions between AM fungi and the rhizoplane bacterial community**

The relationship between bacterial and AM fungal assemblages was explored using the presence or absence profiles of OTUs from the same samples. The semivariance of PCO scores of one microbial taxon was grouped according to the similarity of the OTU presence or absence profiles of the other. This gave a measure of
intersample bacterial (or AM fungal) assemblage variability as a function of associated fungal (or bacterial) assemblage. Principal coordinate semivariance of bacterial assemblages in the second, third and fifth dimensions showed a relationship to AM fungal assemblage OTU profile similarity, regardless of separation distance (up to 6 m) or vegetation composition (Fig. 3A and B). Other PCO dimensions did not show any significant relationship with AM fungal similarity. The reverse relationship was less clear where the semivariance of AM fungal PCO scores was structured as a function of bacterial only in the second dimension but was not consistent across vegetation profile distance classes (Fig. 2B). In two of the five similarity classes, mean semivariance of sample pairs associated with the most similar bacterial populations was greater than for sample pairs with less similar bacterial populations. This suggests that bacterial assemblage similarity did not have a consistent effect on intersample variance of AM fungal community structure (Fig. 2), indicating no impact of bacterial communities on the AM fungal community structure in first 10 dimensions analysed for this study. To determine whether the observed effect was due to both communities being influenced by extrinsic factors, such as local vegetation composition, space, soil pH, etc., in a similar manner, semivariance was calculated for these PCO dimensions between sample pairs as a function of distance of vegetation composition similarity and AM similarity. Principal coordinate semivariance was consistently lower when AM fungal similarity was higher regardless of separation distance or vegetation similarity (Fig. 3A). These observations show that if samples had similar fungal communities, then the bacterial assemblages were also more similar, but the converse was not true for analysed PCO dimensions.

The relationship between bacterial and AM fungal assemblages was further explored by the Procrustean analysis using the presence or absence profiles of OTUs from the same samples. The degree of concordance between the two microbial assemblages was investigated by partial PROTEST (Peres-Neto and Jackson, 2001). This was performed in order to partition out the variation due to the common relationship that the microbial assemblages had with plant communities. The Procrustes rotation using the first 30 PCO dimensions of both bacterial and fungal datasets and 1000 permutation showed that there was a significant concordance (m12 = 0.30; \( P < 0.001 \)) between two assemblages. The residuals were loosely, but significantly, negatively correlated with soil pH (\( r = 0.42; \ P = 0.01 \)), suggesting that the relationship between bacterial and fungal assemblages may be influenced to a small degree by the soil pH. In the present study, we did not investigate bacterial or fungal identity; however, phylogenetic analysis of the bacterial community at the same experimental site revealed that clone libraries were dominated by alphaproteobacteria (~40%) and acidoproteobacteria (~8%; McCaig et al., 1999). Later, Vandenkooomhuysen and colleagues (2002) reported a high diversity of AM fungi from this site as compared with woodland and arable soils. They found 24 different phylotypes, all of which belonged to order Glomales. Phylogenetic analysis revealed that 19 of these phylotypes belonged to the Glomaceae, 3 to the Acaulosporaceae and 2 to the Gigasporaceae. Interestingly, a recent laboratory study with an individual AM fungus G. mosseae has reported a direct influence of the fungus in shaping bacterial assemblages (Artursson et al., 2005), which is consistent with our results. However, our results suggest that such interactions occur at community level in natural conditions rather than at individual AM fungal species level, and emphasize that a complex and multidirectional relationship exists between plant, AM fungi and bacteria. This observation explains why, in field studies, relationships between individual plant species and rhizosphere/ rhizoplane microbial assemblages appear weak (Girvan et al., 2003; Nunan et al., 2005; de Ridder-Duine et al., 2005). Previously it was reported that ecological linkages exist between above- and below-ground biota (Porazinska et al., 2003; Wardle et al., 2004), and that AM fungi play an important role in determining plant biodiversity (Van der Heijden et al., 1998a,b). Our study suggests that AM fungi also affect the bacterial assemblages associated with grass roots, but not the other way round. These results demonstrate that different microbial assemblages respond differently to ecological drivers such as plant diversity, space and pH but, at the same time, interact with each other. Arbuscular mycorrhizal fungal assemblage were linked to both individual plant species and the diversity of plant community, while the influence of individual plant species on their rhizoplane bacterial assemblage is, at most, weak and the interaction is mainly occurred at the community level.

Experimental procedures

Field site, DNA extraction and root identification

Plant material was collected at Fasset Hill, Sourhope (UK National Grid Reference NT 852207), an upland grazed grassland. This site has brown forest soils belonging to the Sourhope series and is derived from old sandstone. The soil at the site has a pH ranging between 3.9 and 5.2 and an organic matter content of 36%. Further details of the soil chemistry are given by Grayston and colleagues (2004) and Ritz and colleagues (2004). The vegetation at the site is a permanent Festuca ovina—Agrostis capillaris—Galium saxatile grassland (unimproved grassland designated U4a by the National vegetation Classification, Rodwell, 1992). The
experimental site was grazed by sheep during the summer months but received no N, P or K fertilizer. Forty spatially referenced soil cores (10 cm diameter, approximately 8 cm depth) were taken from a 14 × 12 m plot. A block of approximately 1 cm² was removed from the centre of the organic layer of each soil core, and 24 active (white coloured) root fragments were randomly selected. Each grass root fragment was approximately 1 cm in length and less than 1 mM in diameter. An average of 14 (range 9–21) fragments from each core were used for further analysis. DNA from individual root fragments was extracted and purified with the Qiagen DNeasy 96 Plant Kit (Qiagen, Cologne, Germany). Each root fragment was identified to genus or species level by PCR-RFLP analysis of the plastid leucine transfer RNA gene (trnL UAA) intron (Ridgway et al., 2003). Roots that could not be identified or from which no PCR product could be obtained for any of the three taxa, i.e. plant, bacteria and AM fungi, were not used in the present study, nor were rare plant species of which only one or two root fragments were identified. In total, 314 samples successfully produced PCR products for all three taxa and were used for further analyses.

Terminal restriction fragment length polymorphism (TRFLP) analysis of the bacterial community from amplified DNA

For TRFLP analysis, general bacterial 16S RNA gene primers were used: forward primer 63F-FAM (5′-AGG CCT AAC ACA TGC AAG TC-3′) and reverse primer 1087R-HEX (5′-CTC GTT GCG GGA CTT ACC CC-3′), 5′-labelled with the fluorochromes 6-FAM (5-carboxyfluorescein) and HEX (hexachlorofluorescein) respectively, and synthesized by Oligo Qiagen (Germany).

The standard PCR reaction mixture contained, in a total volume of 100 µl: 1 × NH₄ reaction buffer (Bioline, London, UK), 1.5 mM MgCl₂, 0.25 mM of each dNTP, 5 pmol of each forward and reverse primer, 20 µg of bovine serum albumin (Roche Diagnostics, Lewes, UK), 2.5 U Biotaq DNA polymerase (Bioline) and 1 µl (roughly 10 ng) DNA template. Polymerase chain reaction (PCR) cycling conditions were: one cycle of 5 min at 95°C; 45 s at 95°C, 45 s at 55°C, 160 s at 72°C (30 cycles); 10 min at 72°C for extension. The PCR products were then purified by ethanol precipitation. Initially, four different restriction enzymes (Hhal, Rsal, Mvnl and TaqI) were used for 25 randomly selected samples to determine the best enzymes for further experiments. Hhal produced more peaks and gave higher consistency, so all further samples were digested only with Hhal. Each 20 µl of reaction mixture contained purified DNA (10 µl), 1 × restriction buffer, 20 µl Hhal (Promega, Southampton, UK) and 0.2 µg acetylated BSA. The volume was made up of autoclaved Milli-Q water. Digestion was carried out at 37°C for 3 h, and the reaction was stopped by incubating samples at 95°C for 15 min. Digested samples to be analysed were mixed with formamide digestion was carried out at 37°C for 3 h, and the reaction was stopped by incubating samples at 95°C for 15 min. Digested samples to be analysed were mixed with formamide and Applied Biosystems GS500 size standard as described in the manufacturer’s instructions and run under standard conditions on an ABI 3700 automated sequencer. Fragment size analysis was carried out using GeneMapper 3.2 (ABI) to define band size and distribution, followed by conversion to presence/absence for statistical analysis.

Statistical analysis

Statistical analysis of the samples was based on the complete sample profiles as expressed by the pattern of TRFLP bands. The analysis consisted of three stages. First, the similarity between every pair of samples was derived. This was calculated as the proportion of shared terminal fragments. Second, the similarities were converted to PCO scores. Principal coordinate scores were then used as the variables to determine the effect of plant root species, spatial location and pH on microbial community structure. Principal coordinate scores were grouped according to plant root species, and differences among groups were determined using REML. The relationship with pH was determined by regression analysis. Patterns in community composition as a function of spatial separation and of the vegetation profiles (relative abundance of plant species) of the cores from which root fragments were sampled were quantified using a semivariogram.
approach. For the latter, rather than using the physical distance among cores to calculate the semivariograms, distance was measured as vegetation dissimilarity or distance (1-ecological coefficient of similarity). Thus, the more similar the vegetation profiles of pairs of cores, the smaller the vegetation distance between the cores. The relationship between bacterial and fungal community structure was measured using the same approach (Nunan et al., 2005). In order to assess the significance of semivariance values for each bin, a bootstrapping procedure was used. Sample scores were randomly re-sampled (with replacement) and a new semivariogram was constructed. As this sampling was random, the semivariograms did not exhibit trends with distance. This procedure was repeated 1000 times and 95% confidence intervals were produced. Semivariance values below the confidence intervals, i.e. those that indicate lower confidence intervals were produced. Semivariance values below the confidence intervals, i.e. those that indicate lower intersample variance than would be expected if patterns did not exist, were significantly \( P < 0.05 \) autocorrelated, while semivariance values within the confidence envelopes were not (Nunan et al., 2005).

Procrustean analysis was chosen over the more commonly used Mantel test to examine the relationship between AM fungal and both plant and bacterial communities, because it is as powerful or more so for detecting matrix associations particularly when the number of observations is high as is the case here (Peres-Neto and Jackson, 2001). A permutation procedure for Procrustean rotation (PROTEST; Jackson, 1995) was used to evaluate the significance of the association between vegetation, AM fungal and bacterial data matrices obtained by TRFLP. The relationship between Procrustean residuals and plant root species was determined by REML and the relationship with pH was determined by regression analysis.

Patterns in community composition as a function of spatial separation of the cores from which root fragments were sampled were quantified by semivariance analysis. All statistical analyses were carried out using the Genstat package (8th edition; VSN International, Hempstead, UK), except for the Procrustean analysis, for which we used the ‘Vegan: Community Ecology Package’ in R version 2.5.1 (the R Foundation for Statistical Computing, 2007).

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References


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