RESEARCH ARTICLE

Community analysis of arbuscular mycorrhizal fungi and bacteria in the maize mycorrhizosphere in a long-term fertilization trial

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Abstract

In this study, we investigated the impact of organic and mineral fertilizers on the community composition of arbuscular mycorrhizal (AM) fungi and bacteria in the mycorrhizosphere of maize in a field experiment established in 1956, in south-east Sweden. Roots and root-associated soil aggregates were sampled four times during the growing season in 2005, in control plots and in plots amended with calcium nitrate, ammonium sulphate, green manure, farmyard manure or sewage sludge. Fungi in roots were identified by cloning and sequencing, and bacteria in soil aggregates were analysed by terminal-restriction fragment length polymorphism, cloning and sequencing. The community composition of AM fungi and bacteria was significantly influenced by the different fertilizers. Changes in microbial community composition were mainly correlated with changes in pH induced by the fertilization regime. However, other factors, including phosphate and soil carbon content, also contributed significantly to these changes. Changes in bacterial community composition and a reduction in bacterial taxon richness throughout the growing season were also manifest. The results of this study highlight the importance and significant effects of the long-term application of different fertilizers on edaphic factors and specific groups of fungi and bacteria playing a key role in arable soils.

Introduction

Soil microorganisms play important roles in determining soil fertility and plant health. Mycorrhizal associations significantly enhance plant nutrient uptake, and soil bacteria either alone, or in concert with mycorrhizal fungi, may contribute to the bioavailability of nutrients in soils (Vessey, 2003; Johansson *et al*., 2004). In conventional agriculture, the application of large quantities of mineral fertilizers represents a disturbance to the soil, which may have profound effects on soil microorganisms (see reviews by Gosling *et al*., 2006; Johansson *et al*., 2004). Long-term application of mineral nitrogen (N) under field conditions has been shown to lead to reductions in mycorrhizal colonization of roots, soil microbial activity and diversity and biomass of soil animals (Mäder *et al*., 2002). Although there are strong indications that high levels of mineral fertilization may affect some microorganisms negatively, most studies have mainly concerned the quantitative changes, e.g. decrease in the level of mycorrhizal colonization (Jensen & Jakobsen, 1980; Mäder *et al*., 2000), sporulation of arbuscular mycorrhizal (AM) fungi (Jensen & Jakobsen, 1980), development of AM fungal extraradical mycelium (Gryndler *et al*., 2001) and microbial biomass and activity (Fliessbach & Mäder, 2000; Mäder *et al*., 2000, 2002; Johnson *et al*., 2005). These quantitative changes can influence plant production and resistance to soil-borne pathogens (Newsham *et al*., 1995). However, qualitative changes in the composition of soil microbial communities may also have profound effects on plant performance. It has been demonstrated that plant diversity, as well as plant nutrient capture and productivity, may increase significantly with increasing species richness of AM fungi (van der Heijden *et al*., 1998). The close relationships between above- and belowground processes also suggest that disturbances affecting soil microorganisms may have an impact on plants.

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Monitoring of changes in the composition of microbial communities, induced by long-term exposure to different fertilization regimes, can therefore be an important step towards sustainable soil management in agriculture.

Some studies based on spore inventories have shown that crop monocultures and heavy application of mineral N may significantly reduce the abundance of some AM fungal taxa (Oehl *et al.*, 2003, 2004). Similarly, long-term mineral fertilization experiments have been shown to have qualitative effects on soil bacterial communities (Hartmann & Widmer, 2006). One such long-term experiment in Sweden, established in 1956, (Kirchmann *et al.*, 1994) was selected for the present study. The site has been characterized previously with respect to denitrifying communities (Enwall *et al.*, 2005) and ammonia-oxidizing bacteria (Nyberg, 2006) in the bulk soil following crop harvest. These studies showed that there was an overall lower activity, in terms of basal respiration, potential denitrification and ammonia oxidation, in plots amended with ammonium sulphate or sewage sludge. In addition, community profiling [by PCR-terminal restriction fragment length polymorphism (TRFLP) and denaturing-gradient gel electrophoresis (DGGE)] revealed that the structure of total bacterial communities as well as denitrifying and ammonia-oxidizing communities was also significantly different in these treatments, and it was suggested that pH is an important factor influencing both the microbial activity and the functional diversity in the bulk soil of this long-term field experiment. In the same field site, Sessitsch *et al.* (2001) analysed bacterial communities in soil fractions of different particle sizes and detected a distinctive bacterial community composition associated with the sewage sludge treatment.

The aim of the present study was to examine how long-term applications of different organic and mineral fertilizers influence the top soil community composition of AM fungi and the bacteria in the mycorrhizosphere, which is defined here as the zone of soil surrounding both the root (rhizosphere) and the extraradical mycorrhizal fungal hyphae emanating from them (Rambelli, 1973; Johansson *et al.*, 2004). An additional aim was to characterize temporal changes in these communities over the course of a growing season, as well as to investigate possible effects of crop harvest on specific groups of bacteria. On the basis of the results of the studies described above, we hypothesized that mineral fertilization would alter the community composition of AM fungi and soil bacteria. In previous experimental studies, we demonstrated a strong relationship between AM fungal hyphal vitality and the degree to which certain bacterial strains could attach to these hyphae (Toljander *et al.*, 2006). On the basis of these results, we further hypothesized that removal of crops or temporal variation may result in a shift in the community composition of bacteria.

Materials and methods

Site description

A long-term field experiment, established in 1956, located in Uppsala (60°N, 17°E, 14 m above sea level), Sweden, was selected for this study; for a detailed description, see Kirchmann *et al.* (1994). The parent material consists of postglacial clay with illite as the main clay material. The mean annual temperature is 5.5 °C and the mean annual precipitation is 527 mm (measured 1961–1990). The experimental design consisted of 14 amendment treatments, laid out in four replicates, located in separate blocks. The individual plots (2 m × 2 m) were separated by wooden frames submerged into the soil. Six of the treatments were chosen for this study, and abbreviated as follows for ease of labelling: a control without nitrogen (N) fertilizer or organic matter (WOM); 80 kg N ha^{-1} $year^{-1}$ in the form of calcium nitrate, $Ca(NO_3)_2$ (CAN); 80 kg N ha^{-1} $year^{-1}$ in the form of ammonium sulphate, $(NH_4)_2SO_4$ (NHS); green manure (GMA); farmyard manure (FYM); and sewage sludge (SES). GMA and farmyard manure consisted of grass and well-decomposed cattle manure, respectively. Organic amendments were standardized with respect to carbon (C) application (4000 kg C ha^{-1} every second year). The last previous application of organic fertilizer was in the autumn of 2004, the year before the present study. The average annual nitrogen application in form of GMA , FYM and SES was 65, 104 and 231 kg ha^{-1}, respectively. In addition, all treatments were amended with 20 kg P ha^{-1} and 38 kg K ha^{-1} annually. A variety of cereals and oilseed crops have been grown alternately on the site; however, since 2000, only maize (*Zea mays* L.) has been grown there. Maize was sown in all plots on 9 June 2005. The aboveground parts of the plants were harvested on 13 September (Blocks 1 and 2) and on 16 September (Blocks 3 and 4), leaving the soil and roots intact to allow sampling of the roots after crop harvest (Table 1).

Sampling and sample preparation

Samples were collected four times in 2005, viz. 27 July, 18 August, 16 September and 6 October. Close to the centre of each individual plot, a soil core (2.8 cm diameter × 15 cm depth) was taken *c.* 3 cm from a maize plant, using a plastic corer. In the October sampling the plants had already been harvested, but a stump with the intact roots system remained in the soil. Samples were taken in all four of the replicated plots of each treatment. Twenty-four samples were taken at each sampling occasion, except in October, when three GMA plots could not be sampled (i.e. $n = 21$), giving an overall total n of 91 soil cores. All samples, remaining in the plastic cores, were stored in separate plastic bags at 4 °C until further processing the same day. The soil

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Table 1. Soil characteristics in a Swedish long-term field experiment with amendments of different organic and mineral nitrogen fertilizers. Data represent mean values of analysis results* from 2005

Treatment	pH	Tot-C (%)	Tot-N (%)	K-Al (mg 100 g^{-1})	P-Al (mg 100 g^{-1})	Crop yield (kg ha^{-1})
WOM	5.95	1.08	0.11	21.45	17.55	3060
CAN	6.28	1.31	0.13	15.63	15.50	6000
NHS	3.85	1.18	0.13	18.25	15.25	1730
GMA	5.73	1.54	0.16	37.20	19.65	5870
FYM	5.93	2.07	0.21	45.65	38.65	6310
SES	4.63	2.60	0.27	13.08	49.90	6030

*Courtesy of Lennart Matsson (Department of Soil Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden).

Soil parameter abbreviations: Tot-C, total carbon; Tot-N, total nitrogen; K-Al, ammonium-lactate extractable potassium; P-Al, ammonium lactate-extractable phosphate.

All figures are calculated on dry weight.

core of each sample was broken apart and roots were picked with sterile forceps and shaken, so that any loose soil would detach from the roots. The remaining aggregates (1–5 mm in diameter) that were firmly attached to the roots were collected using sterile forceps. Roots and aggregates were separated into sterile 15 mL centrifuge tubes, which were then stored at $-20\,^{\circ}$C.

DNA extraction from roots for AMF community analysis

Roots from the July and the August samplings ($n = 48$) were thoroughly rinsed in tap water and cut into lengths of 1 cm. DNA was extracted using a DNeasy Plant Kit (Qiagen, Crawley, UK). For each sample (one per plot and sampling time), a total length of 6 cm of root material was placed in a 2-mL screw-capped propylene tube with half of the volume filled with 2.5 mm zirconia-silica beads (Biospec Products, Bartlesville, OK). The tubes were subsequently filled with 700 μL AP1 buffer from the DNeasy Plant Kit and shaken in a Mini-BeadBeater (Biospec Products) at 5500 r.p.m. for 20 s, kept on ice for 1 min and then shaken again using the same conditions.

PCR, cloning and sequencing of the fungal 18S rRNA gene

For PCR amplifications, the universal eukaryotic primer NS31 (Simon *et al*., 1992) was used as the forward primer. As reverse primers, a mixture of three different primers was used: the AM1 primer (Helgason *et al*., 1998) and the AM2 (5′-GTT TCC CGT AAG GTG CCA AA-3′) and AM3 (5′-GTT TCC CGT AAG GTG CCG AA-3′) (Santos-González *et al*., 2007). The AM1 primer was originally designed as AMF specific, but is frequently mismatched with sequences in *Glomus* group B and group C, and does not target the divergent families *Paraglomeraceae* and *Archaeosporaceae*. The primers AM2 and AM3 are designed to match the same priming site as AM1 in taxa belonging to *Glomus* group B and group C, respectively, as defined by Schwarzott *et al*. (2001). This primer combination amplifies a 550-bp long sequence in the 18S rRNA gene. The puRETaq™ Ready-To-Go™ PCR amplification kit (Amersham Biosciences, Piscataway, NJ) was used with a final reaction volume of 25 μL. Each reaction contained 2 μL of extracted DNA as a template. The thermocycling program used was as follows: 95 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min 30 s. The program was terminated by heating the samples at 72 °C for 10 min. PCR products were cloned with the Invitrogen TOPO-TA Cloning Kit (Invitrogen, San Diego, CA). A minimum of eight clones per clone library was reamplified, using the same primers as in the first PCR. Reamplified fragments were assessed in 0.6% (w/v) agarose gels and those of the expected size were sequenced after being purified with QIAquick (Qiagen, Hilden, Germany). The NS31 primer was used as the sequencing primer in an ABI 3100 Sequencer (Applied Biosystems, Foster City, CA). Sequences were identified using GenBank (http://www.ncbi.nlm.nih.gov/), and were subsequently registered in the EMBL database under the accession numbers DQ497017–DQ497069 for AM fungi and EF474172 –EF474251 for non-AM fungi.

Phylogenetic analysis of fungal sequences

A search for sequences similar to the ones from this study was carried out with the BLAST tool (Altschul *et al*., 1997) provided in GenBank. Sequences with high similarity to AM fungi, together with sequences from cultured AM fungal taxa including representatives of the major groups described by Schwarzott *et al*. (2001), were downloaded. A multiple alignment was assembled using the CLUSTALX program (Thompson *et al*., 1997). The result was refined by eye using the sequence alignment editor Se-Al (Rambaut, 1996). *Endogone pisiformis* Link, a species of *Zygomycota*, which is a possible sister group of *Glomeromycota* (Tehler *et al*., 2003), was used as an outgroup. The region with the most

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variation and thus with the most relevant phylogenetic information lies *c*. 70–300 bp from the 5′ end of the amplicon. This region was used in the following steps of the phylogenetic analysis. The final alignment consisted of 277 aligned positions and 106 terminals.

Maximum parsimony as implemented in the program T.N.T. (Goloboff *et al*., 2003) (Windows version) was used to analyse phylogenetic relationships (Fig. 1). A traditional heuristic search was performed using multiple random addition sequences (10 replicates), followed by branch swapping, using the tree bisection recognition (TBR) algorithm (no more than 10 trees were saved per replicate). Branch support was assessed by performing a jackknife analysis with 1000 pseudoreplications (removal probability = 0.36), using the same parameters as above for tree searching. The clones were grouped into several sequence types that were named after closely related and described species or clones named after the nomenclatural system used by others (Helgason *et al*., 1999; Santos-González *et al*., 2007). The same procedure was followed to analyse sequences that did not belong to the phylum *Glomeromycota*. In this case, the alignment had 280 aligned positions and 109 terminals (see supplementary Fig. 1S)

DNA extraction and PCR TRFLP of the bacterial 16S rRNA gene from root-associated soil aggregates

Frozen soil aggregates were homogenized and mixed using a sterilized metal pestle. From each mixed sample, 500 mg of soil was weighed and transferred into separate 2-mL screw-capped propylene tubes. Total DNA was extracted from the soil using the FastDNA® SPIN Kit for Soil (Q-Biogene Inc., Irvine, CA). PCR of the bacterial 16S rRNA gene extracted from soil aggregates was performed in 40 μL reaction volumes and the final concentrations of constituents in the reaction mix were as follows: 0.2 mM of all four nucleotides, 0.1 μM of each primer, 1.5 mM $MgCl_2$ and 0.025 U μL^{-1} of ThermoRed DNA polymerase (Saveen & Werner, Malmö, Sweden). For the template, DNA extracted from soil aggregates was diluted 1 : 1000 and added as 50% of the total reaction volume. In the TRFLP analysis, the primers 27f (5′-GA GTT TGA TCC TGG CTC AG-3′) (Lane, 1991) and 534r (5′-ATT ACC GCG GCT GCT GG-3′) (Muyzer *et al*., 1993), each labelled in the 5′ end with WellRED dyes D3-PA and D4-PA, respectively, were used. The thermocycler program started with denaturation at 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 60 s, and ended by a final extension at 72 °C for 7 min. DNA concentrations were standardized by visually comparing the amounts of PCR products on 1% w/v agarose gel. PCR products were digested at 37 °C according to the manufacturer's specifications using the restriction endonucleases CfoI and HaeIII (Promega Corporation, Madison, WI) separately. The TRFLP profiles were analysed with a Beckman Coulter CEQ™ 8000 Genetic Analysis System using the CEQ™ DNA Size Standard Kit-600 (Beckman Coulter, Fullerton, CA).

Terminal restriction fragment (TRF) reference database

Four groups were assigned on the basis of a preliminary ordination analysis as follows: (1) WOM, CAN, GMA and FYM sampled in July–September; (2) NHS and SES sampled in July–September; (3) WOM, CAN and FYM sampled in October; and (4) NHS and SES sampled in October. In order to obtain representative clones from each of the four sample groups, cloning was performed separately within each group. Samples were amplified under the same conditions as above, using the primers 27f and 1492r (5′-TACGGY-TACCTTGTTACGACT-3′). The resulting PCR products were pooled within the four respective groups. The concentrations of the original PCR products and of the pooled products were measured and standardized so that equal amounts of DNA template were used for each cloning reaction (*c*. 20 ng DNA per ligation reaction). PCR products were cloned using the Invitrogen TOPO-TA Cloning Kit (Invitrogen). From each of the four cloning reactions, 32 clones with inserts were reamplified and sequenced using primers m13f and m13r provided in the TOPO-TA cloning kit. Sequences were analysed using a Beckman Coulter CEQ™ 8000 Genetic Analysis System. SeqMan™ version 5.07 (DNASTAR, Madison, WI) was used for sequence editing. Sequences were quality trimmed at their 3′ and 5′ ends to a common 500-bp fragment, blasted against GenBank and subsequently registered in GenBank under the accession numbers EF474252–EF474342. A subset of these clones, representing only those bacterial taxa showing a significant response to either treatment or time or sampling, is presented in Table 4. To obtain phylogenetic descriptions of the clones, sequences were blasted and aligned against selected reference sequences from GenBank, using the CLUSTALV algorithm in MEGALIGN version 5.07 (DNASTAR). Reference sequences were selected to present the highest sequence match and the lowest Expect value (usually $e = 0.0$). To obtain TRFLP profiles of the individual clones, nested PCR using WellRED primers was performed on diluted (1 : 500) amplified clone inserts. The procedures for PCR and TRFLP analysis were the same as described in the previous paragraph. All clone sequences were subjected to *in silico* restriction fragment analysis, and showed a high agreement with the TRFLP analysis. In the subsequent matching of environmental TRFLP profiles against the clone libraries, additional comparisons against a clone database from a previous study (Toljander *et al*., 2007) were also used.

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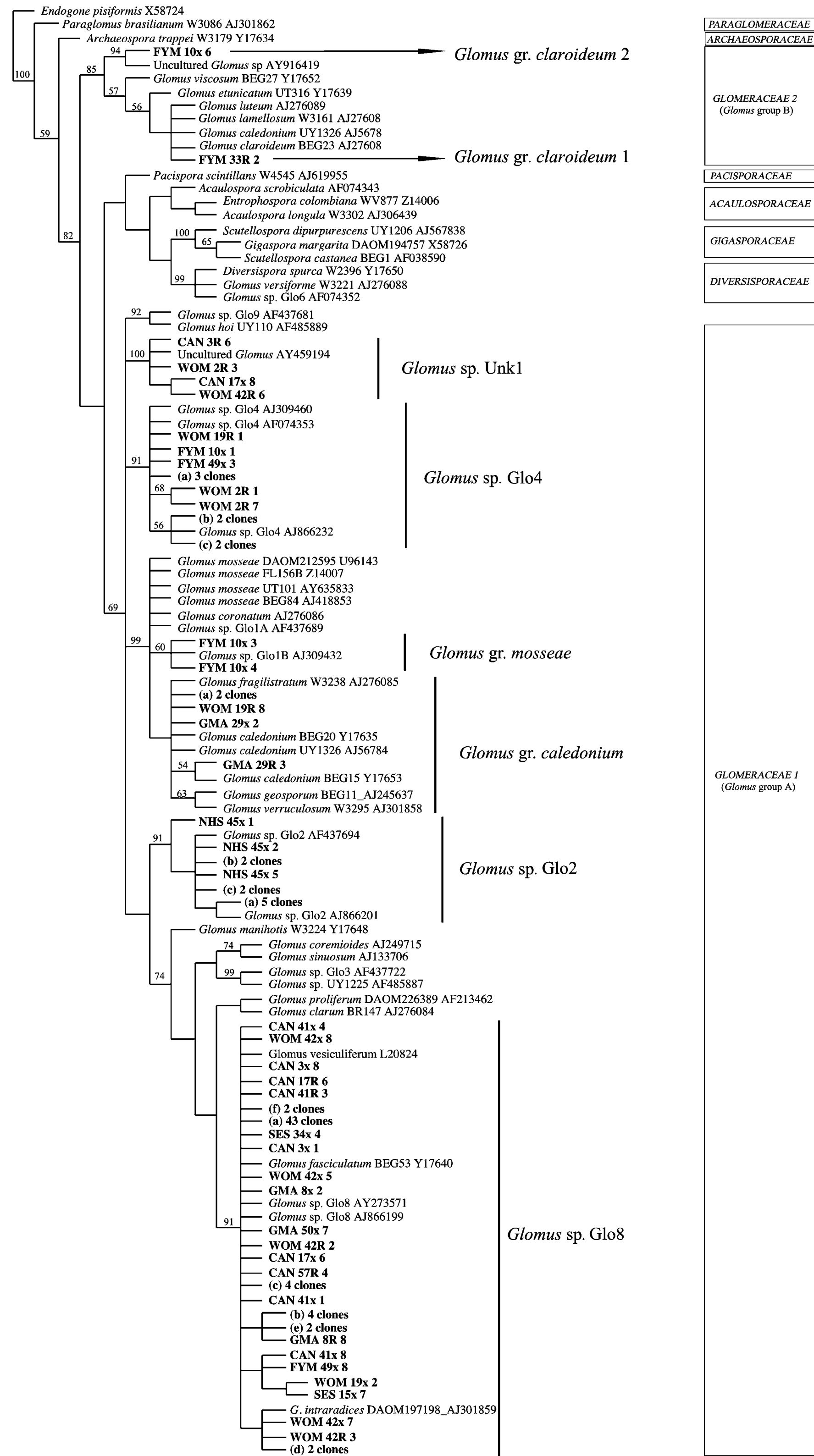

Fig. 1. Strict consensus tree of 30 equally parsimonious trees showing the different sequences of AM fungi obtained from the clone libraries (in bold). *Endogone pisiformis* (phylum *Zygomycota*) was used as an outgroup taxon. Values above branches are from a jack-knife analysis (1000 pseudoreplicates, removal probability = 0.36). The sequence groups on the right (e.g. *Glomus* sp. Glo 8, *Glomus* gr. *mosseae*, etc.) identify sequence groups with sequence similarity > 96% and the boxes identify families. Clone identifiers relate to soil fertilizer treatment, e.g. WOM, CAN, SES; plot number where each sequence group was recorded and time of sampling (x = July; R = August) plus a clone number. Groups of clones with identical sequences are represented by a letter and the number of identical clones (see supplementary Table 1S for a detailed description of clone identifiers).

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These clones originated from no nmanipulated field soil close to the experimental plots of the present study. As a result, potential match was studied between the environmental TRFLP profiles and a total of 150 TRFLP-types (taxa), each associated with a unique TRFLP profile and a GenBank identity.

Identification of unknown bacterial TRFs

To assign TRFs from the unknown samples' putative identities, the TRFLP profiles were compared with profiles in the TRF reference database using TRAMP (Dickie *et al.*, 2002), with the maximum acceptable error for fragment size set to ± 1 bp, and the threshold parameter was set to exclude fragments with a peak area (signal intensity) of $< 3\%$ of the highest peak area from the analysis.

Statistical analysis

Two-way ANOVA in Minitab® release 14 (Minitab Inc., State College, PA) was used to examine the possible significant ($P \leq 0.05$) influence of treatment, time of sampling, block and the interaction between these factors on the species richness of fungi and bacteria. Further analyses using χ^2 tests ($P \leq 0.01$) were performed to test the hypothesis of equal distribution of fungal and bacterial taxa in the different sample categories (treatment; time of sampling). For bacteria, three plots in the GMA treatment could not be sampled in October. However, omitting the GMA treatment from the analysis altogether, or overestimating taxon occurrence by adding one observation per taxon for each missing sample, did not significantly influence the general outcome of the analysis. For each of the taxa that exhibited a strongly significant nonrandom distribution, Fisher's exact test ($P \leq 0.05$) in Minitab was performed in pair-wise comparisons to determine differences in taxon distribution between separate pairs of treatments or time points of sampling. In order to control the overall error rate to 0.05, the significance level of a single pairwise comparison was set to $0.05/15 = 0.0033$ for treatments and to $0.05/6 = 0.0083$ for time points because 15 and 6 comparisons were needed for the six treatments and four time points, respectively.

The frequency of occurrence of each taxon was recorded in terms of presence (1) or absence (0) in samples. A relative frequency of occurrence of TRFs or taxa within every treatment was calculated by adding the values from the replicated plots within each treatment at each time point of sampling (July, August, September, October) and then dividing by the number of replicated plots. The values of relative frequency of occurrence were arcsine-transformed before ordination analysis. To analyse the potential influence of environmental variables and time of sampling on the bacterial community composition, correspondence analysis (CA) and canonical correspondence analysis (CCA) with Monte–Carlo permutation tests ($n = 999$) were performed on the species data using Canoco for Windows version 4.52 (ter Braak & Smilauer, 1998). In the CCA, a full model including all variables was used to test the significance of the relationship between these environmental variables and the bacterial community data, and the significance of each variable was in addition tested individually.

A Mantel test with PC-ORD version 4.25 (McCune & Mefford, 1999) was applied to test whether there was a correlation between the composition of the bacterial and the fungal assemblages across the different treatments. Incidence matrices (presence/absence) for bacteria and AM fungi were calculated by combining the results of the samples taken in July and August. Two similarity matrices of identical dimensions (24×24) were generated for both assemblages, using the classical Sørensen incidence-based index as it is implemented in ESTIMATES version 7.5 (Colwell, 2005). The significance of the standardized Mantel statistic (r) was evaluated with a randomization (Monte–Carlo) test (1000 permutations).

Results

Phylogenetic analysis of AM fungal sequences and community composition

From the 285 sequences obtained from the clone libraries, 115 sequences (40%) belonged to AM fungi. AM fungal sequences were recorded in all treatments but not in all plots. The maximum parsimony analysis retained 30 trees as short as 677 steps. The most frequently represented taxonomical group in our analysis, the putative family *Glomeraceae* 1 (*Glomus* Group A), received 88% support, while Glomeraceae 2 (*Glomus* Group B), *Gigasporaceae* and *Diversisporaceae* received 91%, 100% and 99%, respectively. Consequently, *Glomeraceae* in its present circumscription is rendered paraphyletic in our analysis, because *Acaulosporaceae*, *Gigasporaceae* and *Diversisporaceae* are nested within it. The newly obtained sequences from our study clustered within two putative families called *Glomeraceae* 1 (*Glomus* Group A) and *Glomeraceae* 2 (*Glomus* Group B) by Schüßler *et al.* (2001). The sequences were grouped into eight discrete sequence types (taxa) with an intragroup pairwise similarity $> 96\%$. These sequence groups represent eight putative species belonging to the paraphyletic genus *Glomus*: *Glomus* sp. Glo8 or *Glomus* gr. *intraradices*; *Glomus* sp. Glo 4; *Glomus* sp. Glo 2; *Glomus* gr. *caledonium*; *Glomus* sp. Glo unk8; *Glomus* gr. *mosseae*; *Glomus* sp. Glo unk6; and *Glomus* sp. Glo unk7 (Table 2, Fig. 1). All but the sequence groups *G.* gr. *caledonium* and *G.* gr. *mosseae* sequence groups receive significant or near -significant support ($> 90\%$) in the jackknife analysis. The sequences clustering within the species complex formed by *G. mosseae*, *G. caledonium* and related

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Table 2. Sequence groups of AM fungi found in maize roots in a Swedish long-term field experiment with amendments of different organic and mineral nitrogen fertilizers

Sequence group	WOM*	CAN	NHS	GMA	FYM	SES
Glomus gr. *intraradices*	2	4		2	1	2
Glomus sp. Glo4	2	1		3	1	
Glomus sp. Glo2			1			
Glomus gr. *caledonium*	1			2		
Glomus sp. Glo unk8	2	2				
Glomus gr. *mosseae*				1		
Glomus gr. Glo unk6				1		
Glomus gr. Glo unk7				1		
Richness	4	3	1	6	2	1

*PCR amplification was only successful in two samples of the WOM treatment in July.

Numbers represent the number of replicated plots per treatment where they were detected, i.e. '4' denotes that a group was detected in all four replicated plots.

taxa were assigned to two different groups albeit with very low support. In this group, the species delimitation is problematic and high levels of intra-specific variation in the rRNA gene have been reported before (Clapp *et al.*, 2001). *Glomus* sp. Glo8 or *G.* gr. *intraradices*, hereafter referred to as *G. intraradices*, was the AM fungal taxon with the highest representation in the clone libraries (68% of all AM fungal sequences). It was represented in all treatments, except in that with ammonium sulphate. In 42% of all plots (28% of all samples), *G. intraradices* was the only AM fungus detected. The AM fungal community did not change significantly between different sampling times. There was variation in community composition between different fertilization treatments (Table 2); however, the only significant effect ($P < 0.05$) was that on *G. intraradices*. In addition, two- to sixfold higher ($P < 0.01$) numbers of clones representing *G. intraradices* were recorded in the calcium nitrate treatment, compared with any other treatment where this AM fungus was found. The highest AM fungal richness was recorded in the green manure treatment (Table 2).

Detection of non-AM fungi in roots

From the 285 sequences obtained from the clone libraries, 167 sequences (59%) belonged to the phylum *Ascomycota*. One taxon belonging to *Chytridiomycota* (100% similarity to *Olpidium brassicae* DQ322624) was found in two single plots. In the phylogenetic analysis, the ascomycete sequences grouped together with known taxa belonging to four different classes (supplementary Fig. 1S), but an unequivocal assignment to known Ascomycete species was not possible. The number of ascomycete sequences detected among clones was strongly influenced by fertilization treatment ($P \leq 0.01$), the greatest difference being that fewer ($P < 0.05$) ascomycete sequences were detected in plots with calcium nitrate and without organic material, compared with the four other treatments.

Bacterial community composition

Cloning and sequencing yielded in total 90 unique bacterial sequence types (taxa), and the mean number $\pm$ SE, of bacterial taxa identified per sample was 28 ± 2. Most of these taxa had a uniform distribution among samples, or were only detected in a very small number of samples, and were therefore not included in the discussion below. There was a strong effect of time of sampling ($P \leq 0.001$), but not of treatment ($P = 0.142$) on taxon richness (Fig. 2a and b). Overall, taxon richness decreased between July and October. Taxon richness, but not community composition, was significantly ($P < 0.05$) related to the plot location in the randomized block design. In general, the taxon richness was lower in samples from Block 2 (Fig. 2c).

While fertilizer treatment did not significantly affect taxon richness, it did, however, influence the frequency of occurrence of some individual bacterial taxa. Based on ordination analyses and χ^2 tests, a total of 12 taxa displayed a strongly significant ($P \leq 0.01$) nonrandom distribution in samples (Table 4). Seven of these were influenced by fertilizer treatment. The occurrence of the remaining five taxa was influenced by the time of sampling. Most of these taxa showed a declining trend between the four different sampling occasions. None of these taxa had a skewed distribution among the different blocks of the randomized block design.

The use of two fluorescently labelled primers and two restriction endonucleases resulted in four TRFLP profiles representing each sample. Because the outcome of the ordination analysis was not influenced by the choice of profile, D4-PA-labelled TRFs of the restriction analysis using CfoI were chosen to represent the bacterial community in the samples in the preliminary, unconstrained ordination analysis (Fig. 3a). In the scatter plot resulting from the CA, the bacterial communities separated into different groups, generally corresponding to the respective treatments (Fig. 3). The bacterial communities in the control treatment without fertilizer amendments clustered in the centre of the plot. The ordination shows that the community structures of the green and the farmyard manure treatments are more similar to the controls than the other treatments. Samples from the ammonium sulphate and the sewage sludge plots were markedly separated from the other treatments along the first axis, and ammonium sulphate and sewage sludge were in addition separated from each other along the second axis. Sewage sludge and calcium nitrate showed a similar distribution along the second axis. There was, in addition, a general trend for treatments to separate along the second

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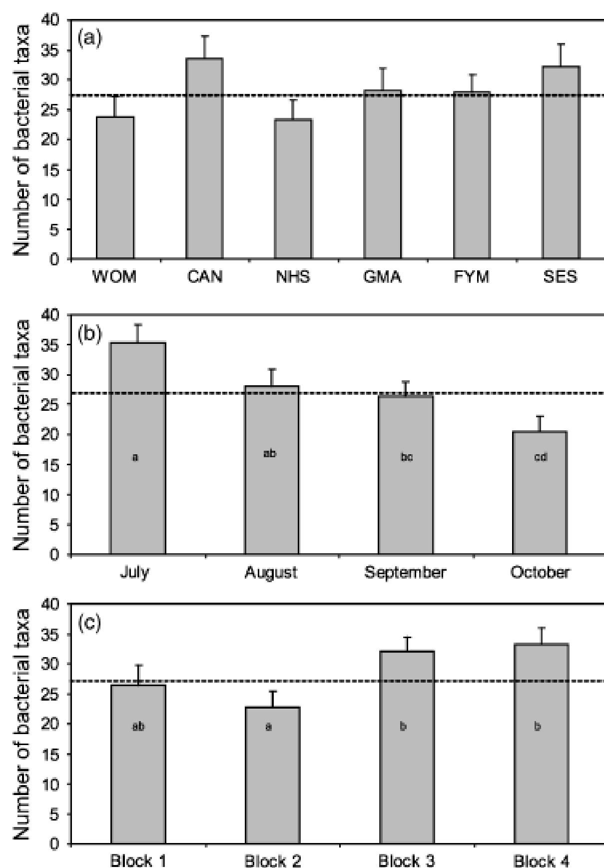

Fig. 2. Average numbers of bacterial taxa ± SE of the means detected by PCR TRFLP and DNA sequencing of clones from root-associated soil aggregates from a long-term field experiment with amendments of different organic and inorganic fertilizers. Each fertilizer treatment was represented by four replicated plots, and was sampled four times each during the growing season. The diagrams represent samples in the different categories (a) Fertilizer treatment, (b) Time of sampling and (c) Block number, i.e. plot location in the randomized block design. The dashed line represents the overall mean number (28.2) of taxa, irrespective of the sample category. Significant differences ($P < 0.05$) from two-way ANOVA are denoted by the letters a, b, c and d. No significant ($P = 0.142$) differences in numbers of taxa with respect to fertilizer treatment were detected. Fertilizer treatments: WOM, without organic material or mineral fertilizer; CAN, $Ca(NO_3)_2$; NHS, $(NH_4)_2SO_4$; GMA, green manure; FYM, farmyard manure; SES, sewage sludge.

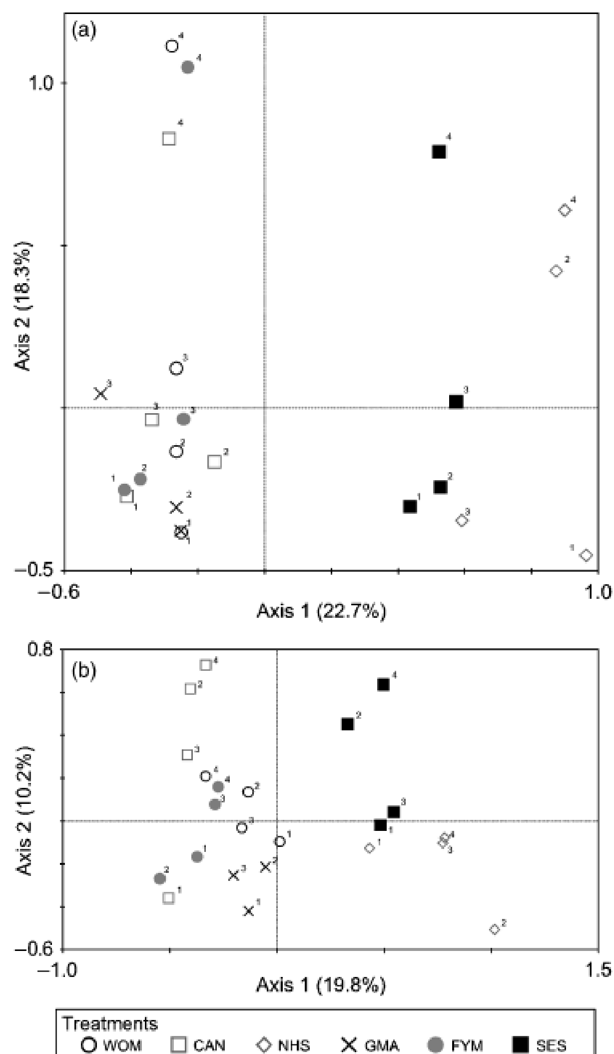

Fig. 3. Scatter plots derived from CA of (a) TRFs and (b) bacterial taxa detected by PCR TRFLP and DNA sequencing of clones from root-associated soil aggregates from a long-term field experiment with amendments of different organic and inorganic fertilizers. Each fertilizer treatment was represented by four replicated plots, and was sampled four times each during the growing season (the only exception was GMA-4, for which data are missing). Numbers denote time of sampling 1, July; 2, August; 3, September; 4, October (post harvest of crop). Fertilizer treatments: WOM, without organic material or mineral fertilizer; CAN, $Ca(NO_3)_2$; NHS, $(NH_4)_2SO_4$; GMA, green manure; FYM, farmyard manure; SES, sewage sludge.

axis, according to time of sampling. In the CA, the total inertia of the species data was 1.036, and the fraction of the total variance in the species data explained by the first and second axes together was 28.9%. In the full model of the CCA, the cumulative variance explained by the canonical axes was 37.6%. Several of the environmental parameters included in the full model were strongly correlated with Axis 1 or Axis 2. The strongest correlation was found between soil pH and the first axis ($r = 0.977$, $P < 0.001$). The second axis was most strongly correlated with extractable phosphate concentration ($r = 0.835$, $P < 0.001$). A summary of all results from the correlation analyses and the CCA with Monte–Carlo Permutation tests is shown in Table 3.

The Mantel test did not detect any significant relationship between the bacterial and the AMF similarity matrices ($r = 0.164$, $P = 0.110$).

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Table 3. Summary of results from CCA with Monte-Carlo permutation tests ($n = 999$) on the bacterial community composition in samples from root-associated soil aggregates collected in a Swedish long-term field experiment with amendments of different organic and mineral nitrogen fertilizers

Variable	Correlation (r) 1st axis	2nd axis	Fraction of total inertia explained by variables	Monte-Carlo test P values for all canonical axes
Full model[1]			0.376	$P < 0.001$
Soil pH	0.977***	– 0.079 (NS)	0.179	$P < 0.001$
Total nitrogen	– 0.353 (NS)	– 0.801***	0.079	$P < 0.05$
Extractable potassium	0.466*	0.231 (NS)	0.078	$P < 0.05$
Extractable phosphorus	– 0.274 (NS)	– 0.835***	0.075	$P < 0.05$
Total carbon	– 0.256 (NS)	– 0.818***	0.072	$P < 0.05$
Crop yield	0.367**	– 0.719**	0.031	$P < 0.05$

[1]The full model includes all data presented in Table 1 as explaining variables.
Asterisks denote level of statistically significant relationship between variables and axes in the CCA:
***$P \leq 0.001$;
**$P \leq 0.01$ and
*$P \leq 0.05$.
NS, not significant.

Table 4. Bacterial taxa detected by PCR TRFLP and DNA sequencing of clones in root-associated soil aggregates from a Swedish long-term field experiment with amendments of different organic and mineral nitrogen fertilizers

Clone no. (acc. no.)	Frequency of occurrence*(%)	Closest NCBI match (acc. no.)	Phylogenetic description	Similarity (%)	Fertilizer treatment† WOM	CAN	NHS	GMA‡	FYM	SES	Time of sampling† Jul	Aug	Sep	Oct
112 (EF474326)	28	Uncult. *Acidobacteriaceae* (EF074820)	*Acidobacteriaceae*	99	5^{bc}	1^{bc}	16^{a}	5^{bc}	0^{c}	9^{ab}	10	10	10	6
095 (EF474319)	28	Uncult. *Gemmatimonadetes* (AY921714)	*Gemmatimonadetes*	97	5	11	4	6	3	7	17^{a}	5^{b}	9^{ab}	5^{b}
016 (EF474265)	27	Uncult. *Acidobacteria* (DQ828544)	*Acidobacteria*	97	3	7	6	9	9	1	17^{a}	9^{ab}	4^{b}	5^{b}
032 (EF474278)	26	Uncult. bacterium (AY218764)	*Gemmatimonadetes*	98	3^{bc}	5^{abc}	10^{ab}	1^{c}	2^{c}	12^{a}	13	5	7	8
054 (EF474292)	26	Uncult. *Acidobacteria* (AF200696)	*Acidobacteria*	98	2^{bc}	0^{c}	15^{a}	6^{bc}	0^{c}	10^{ab}	9	10	8	6
117 (EF474329)	24	Uncult. Firmicutes (EF074729)	Firmicutes	95	1^{b}	2^{b}	12^{a}	4^{ab}	1^{b}	12^{a}	10	9	7	5
025 (EF474337)	22	Uncult. *Rubrobacteridae* (AY150874)	*Actinobacteria*	96	3^{ab}	1^{b}	6^{ab}	3^{ab}	5^{ab}	11^{a}	11	8	7	3
120 (EF474331)	22	Uncult. eubacterium (AF047646)	*Acidobacteriaceae*	98	5^{ab}	0^{b}	10^{a}	10^{a}	0^{b}	3^{ab}	11	7	6	4
071 (EF474305)	19	Uncult. bacterium (AY584744)	*Chloroflexi*	96	3	8	2	3	7	2	12^{a}	4^{ab}	8^{ab}	1^{b}
062 (EF474297)	19	Uncult. betaproteobacterium (AF431269)	*Burkholderiales*	100	3	4	2	4	5	6	13^{a}	5^{ab}	4^{b}	2^{b}
080 (EF474274)	14	Uncult. bacterium (AB128876)	*Acidobacteria*	96	2	4	3	3	1	5	11^{a}	2^{b}	4^{ab}	1^{b}
092 (EF474317)	12	Uncult. *Gemmatimonadetes* (AY395331)	*Gemmatimonadetes*	97	0^{bc}	0^{c}	1^{a}	1^{bc}	2^{c}	11^{ab}	4	4	4	3

*Frequency of occurrence refers to percent of all sampled plots where taxon was detected, irrespective of fertiliser treatment or time of sampling.
†Numbers in columns refer to number of samples in each sample category (fertilizer treatment or time of sampling) where taxon was detected.
Letters *a, b, c* in superscript denote level of statistically significant differences (where applicable) in the distribution of individual taxa among samples, as determined by Fisher's exact test ($P \leq 0.05$).
‡Three plots could not be sampled in October, leading to a possible under-estimation of observations in this treatment.
Table includes only those clones with a strongly significant ($P \leq 0.01$) nonrandom distribution in samples, as determined by χ^2 test for independence. Clones are ordered according to their frequency of occurrence in samples. A complete record of the clone sequences has been submitted to GenBank under accession numbers EF474252–EF474342.

Discussion

Effects of fertilizer amendments on bacterial community composition

Although there were no significant changes in bacterial taxon richness, clear differences in community composition were detected among the six treatments. The CA ordination (Fig. 3b) shows that plots amended with ammonium sulphate and sewage sludge were separated from other treatments in terms of bacterial community composition, which can be explained by the predominance of several bacterial taxa in both the ammonium sulphate and the sewage sludge treatments (Table 4): *Acidobacteria* (Clones 112 and 054), Gemmatimonadetes (Clone 032), Firmicutes (Clone 177) and Actinomycetes (Clone 025). Sessitsch *et al.* (2001) also observed a unique bacterial community in soil amended with sewage sludge, but also found that the soil particle size fraction, from which the bacteria were extracted, had an even stronger impact than the fertilization regime on the bacterial community structure. This may partly be explained by the fact that their study did not include the ammonium sulphate treatment, which, in later studies, was found to harbour one of the most distinct

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bacterial communities (Nyberg, 2006). Similar to our own results, Sessitsch *et al.* (2001) showed that *Acidobacteria* were able to colonize most treatments but, in contrast to the present study, *Acidobacteria* were generally much less common in SES. This inconsistency may be due to the change of crop species between studies, or because Sessitsch *et al.* did not sample rhizosphere soil. A more recent study demonstrated that community profiles of denitrifying communities in the bulk soil following crop harvest in the two low -pH treatments i.e. ammonium sulphate and SES , were significantly different compared with other treatments (Enwall *et al.*, 2005). Community profiles of ammonia -oxidizing bacteria were also different in the bulk soil of these treatments, and in addition the ammonium sulphate and the sewage sludge treatments displayed individually different communities (Nyberg, 2006). In the present study, there was a clear separation of ammonium sulphate from sewage sludge along the second axis of the CA. This, coupled with similar positions of sewage sludge and calcium nitrate, indicates that pH was not solely responsible for the observed changes in the community composition. In particular, extractable phosphorus and crop yield were highly correlated with the second axis. Other factors influencing the microbial community composition in the plots amended with sewage sludge may include high concentrations of heavy metals (Sessitsch *et al.*, 2001) and bacteria added with the sewage sludge itself. In particular, Gemmatimonadetes bacteria (Clones 032, 092 and 095; Table 4) were common in this treatment. This group of bacteria were indeed first described in activated sludge in a sewage treatment system (Zhang *et al.*, 2003). Environmental sequence data published in GenBank suggest that this phylum is widespread in nature. It therefore appears likely that the enrichment of these bacteria in our plots was due to additions through sewage sludge amendment and/or that they comprised indigenous populations that were promoted by the soil conditions resulting from amendment with sludge.

The relationship between the second axis and the bacterial community composition was further investigated by removing the data for relative frequency of occurrence and only using qualitative (presence/absence) data in the CA. This resulted in a similar clustering of treatments along the first axis (not shown) as in the previous analysis (Fig. 3), but with less scattering along the second axis. The positions of samples along the second axis were therefore not only related to the species composition, but were also due to similarities in the relative frequency of occurrence of individual taxa. The largest similarities between the calcium nitrate and the sewage sludge treatments, which had similar positions on the second axis, therefore reside in their higher taxon richness and relative frequency of occurrence of taxa.

Plots amended with organic fertilizers (green and farmyard manure) were, in general, associated with a (nonsignificantly) higher taxon richness than control plots without amendments. A significant part of our present knowledge about long-term effects of organic fertilizers on microbial communities originates from the DOK long-term field trial in Switzerland. Esperschutz *et al.* (2007) showed that farmyard manure application consistently had the strongest influence on microbial diversity and community structure, and that prokaryotic communities responded most strongly to either conventional or organic farming management. Hartmann & Widmer (2006) further attempted to identify treatment-specific indicator taxa in the DOK experiment. In our study, the correlation analysis (Table 4) demonstrated a strongly significant relationship between soil carbon content and the community data. Many of the taxa that were common in the green and farmyard manure treatments also occurred in plots without organic amendments, albeit at a lower frequency of occurrence, and it was therefore difficult to confirm statistically whether individual bacterial taxa were more dominant in a particular treatment. It should also be noted that three plots amended with green manure could not be sampled in October and it is therefore possible that the occurrence of some taxa in this treatment has been underestimated. However, this did not influence the general outcome of the analysis. One *Acidobacteriaceae* bacterium, represented by Clone 120 (Table 4), had a noticeably higher frequency of occurrence in the green manure plots. Because this taxon was also very common in the plots amended with ammonium sulphate (and with a low organic content), it is likely that it was favoured by factors other than those associated with organic amendments only. The lowest bacterial taxon richness was observed in control plots and in plots amended with ammonium sulphate. It was demonstrated previously in this long-term trial that application of ammonium sulphate resulted in a reduction in the ratio of microbial biomass-C to soil-C (Witter *et al.*, 1993; Witter & Dahlin, 1995) and basal respiration (Nyberg, 2006), which can be interpreted as a decline in the total bacterial population density. However, the frequency of occurrence of some individual taxa (mainly representing *Acidobacteria* and Firmicutes) was high in the ammonium sulphate treatment in the present study, compared with all the other amended plots. Even though the ammonium sulphate treatment resulted in a decreased bacterial richness in the present study, the remaining dominating taxa had a high frequency of occurrence, which was comparable to or higher than that of taxa dominating in other treatments. It is, however, important to note that analysis of the relationships between treatments effects and bacterial taxon richness has to be performed with caution. Soil bacterial communities usually exhibit an extreme taxon richness that necessitates intensive sampling

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on a scale that is seldom practically or economically possible.

Effects of fertilizer amendments on AM fungal community composition

The eight sequence groups detected in our study belong to the genus *Glomus*, but grouped into two different families (*Glomeraceae* A and B). Thus, *Glomus* appears to be paraphyletic in our phylogenetic analysis. Using the nearly full length of the 18S rRNA gene, Schwarzott *et al*. (2001) found these two groups to be monophyletic, and suggested that they could be considered to be two separate 'families' (*Glomus* group A or *Glomeraceae* A; and *Glomus* group B or *Glomeraceae* B). It was also suggested that a better understanding of the phylogenetics of *Glomeromycota* is needed before renaming these groups. In our study, we could not detect sequences belonging to the families *Acaulosporaceae*, *Scutellosporaceae* and *Diversisporaceae*, although we were able to detect them in a semi-natural grassland in the same region using the same primer combination (Santos-González *et al*., 2007). Some taxa in these families may be rare or absent in the arable field investigated, but they could also have been missed with our sampling method because of sampling effort, patterns of seasonality or because some taxa may colonize roots deeper in the soil (Oehl *et al*., 2005). As expected, the families *Archaeosporaceae* and *Paraglomeraceae* were not detected because the primer combination used does not target these families. These two families comprise few species, which are difficult to characterize morphologically (Redecker & Raab, 2006) and that are not usually detected with primer combinations aimed to target other groups of *Glomeromycota*. Although some species of *Archaeosporaceae* and *Paraglomeraceae* seem to be widely distributed and can be relatively abundant in certain ecosystems, some studies indicate that they do not appear to be dominant or diverse in arable systems (Oehl *et al*., 2003, 2005; Hijri *et al*., 2006).

Although we studied only one site and we detected many root samples without AM fungi, the overall number of sequence groups is relatively high in comparison with similar studies. In a conventional arable maize field in Germany, Hijri *et al.* (2006) found only three AM taxa, whereas a total of ten sequence groups were found in maize roots in the 'DOK-experiment' in Switzerland. The results are, however, not directly comparable to ours, because Hijri *et al.* (2006) used a different experimental design and also targeted a different region of the AM fungal rRNA gene, using a primer combination that also detects the genera *Paraglomus* and *Archaeospora*. Using the primer combination AM1/NS31, Daniell *et al.* (2001) and Helgason *et al.* (1998) also found 10 sequence types in four different arable sites during a 2-year long survey. However, in their maize field only four taxa were found.

Glomus mosseae and closely related taxa have been reported as common and typical from arable fields (Helgason *et al*., 1998; Daniell *et al*., 2001; Kjoller & Rosendahl, 2001; Oehl *et al*., 2003; Hijri *et al*., 2006; Öpik *et al*., 2006); however, in our study this group of AM fungi was only rarely found in the roots. The second most common sequence group in our study, the morphologically uncharacterized *Glomus* sp. Glo4, was also found to be very common in all arable fields in the study by Daniell *et al.* (2001). As in our case, Hijri *et al.* (2006) found *G. intraradices* to be the most widespread taxon, occurring in all the treatments. The occurrence of *G. intraradices* was significantly affected by the fertilization treatments of the present study. Although this fungus was the overall most frequently recorded species, it was not detected in the ammonium nitrate treatment. In contrast, the addition of calcium nitrate resulted in a massive presence of *G. intraradices* in maize roots. This species has been reported previously as being 'nitrophilic' (Scheublin *et al*., 2004; Jumpponen *et al*., 2005). The effects of mineral nitrogen on AM fungal community composition are not well understood, but many studies indicate that nitrogen fertilization can have a negative impact on the functional structures of AM fungi and on the richness of AM fungi colonizing plant roots (Santos *et al*., 2006)

In the present study, the highest number of AM fungal species was detected in three treatments (control, calcium nitrate and farmyard manure) exhibiting the highest soil pH and intermediate to high levels of total nitrogen (Table 2). The absence of *G. intraradices* and other AM fungal species in the ammonium sulphate, and the fact that only one AM fungal species was detected in both the ammonium sulphate and the sewage sludge treatments, respectively, suggest that low pH may have adversely affected the AMF community in this study. It is also possible that the stunted plant growth related to the amendment with ammonium sulphate may have resulted in plants with an overall lower mycorrhizal colonization, or that the maize roots in this treatment had fewer AM fungal species associated with them. In the sewage sludge treatment, low pH in combination with high phosphorus availability and high soil concentrations of metals may have inhibited AM fungal growth. A number of studies have reported that root colonization by some AM fungi, as well as development of AM fungal mycelia in soil, can be stimulated by amendment of different organic substrates (Gryndler *et al*., 2002, 2005, 2006). In the present study, there was no apparent stimulatory effect of amendments with organic material on the diversity of AM fungi. Although the highest number of AM fungal species was, indeed, recorded in the treatment with green manure amendment, the number of observations for individual sequence types was too low to confirm a statistically significant trend.

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Ascomycete sequences

Sequencing of AM fungal clones unexpectedly revealed that many of the cloned inserts represented ascomycetes. The AM1 primer is frequently used to study AM fungi in roots (Helgason *et al.*, 1998; Husband *et al.*, 2002; Vandenkoornhuyse *et al.*, 2002; Cornejo *et al.*, 2004). This primer can amplify the 18S rRNA gene of many AM fungal families, but it has also been reported to amplify basidiomycetes and ascomycetes (Heinemeyer *et al.*, 2004; Douhan *et al.*, 2005). Probably, the primers AM2 and AM3, which were tested here for the first time on an agricultural field, also show affinity for at least some non-AM fungal sequences. Nevertheless, the total number of AM fungal sequence types detected in the present study was comparable to or even higher than that of similar studies. Additionally, when we used the same primer combinations in a previous study, relatively few ascomycetes were detected in roots of two perennial forbs in semi-natural grasslands (Santos-González *et al.*, 2007). This may indicate that plant roots in agricultural systems are more heavily colonized by non-AM fungi. Using general fungal primers, Gomes *et al.* (2003) found that the fungal community in the maize rhizosphere was dominated by ascomycetes, many of which are often referred to as dark septate endophytes in the literature. The relationship between and dark septate endophytes and plants, and the role of these fungi in the rhizosphere is far from clear (Jumpponen, 2001). Unfortunately, the phylogenetic resolution of the 18S rRNA gene in ascomycetes is much lower than that in *Glomeromycota*. This lack of accuracy in the taxonomic status of the sequences, together with the fact that we do not fully know how well our primers target diverse groups of ascomycetes and other fungi, makes it difficult to provide a reliable picture of the community of ascomycetes. However, Santos-González *et al.* (2007) found seasonal- and plant species-related differences in the colonization of plant roots by ascomycetes and suggested that future studies of the distribution of glomeromycotan fungi should pay more attention to the distribution of other groups of fungi colonizing roots.

Temporal changes and effects of crop on microbial community composition

Within each of the six treatments, there was a separation of bacterial communities along the second axis of the CA with respect to time of sampling. There was also a significant decline in bacterial taxon richness between July and October. This was probably due to a general decrease in the frequency of several taxa, causing very rare taxa to disappear or be excluded from detection. When using the bacterial taxon data (Fig. 3b), there is a consistent, but nonsignificant, trend for October samples, to be shifted upwards along the second axis, compared with other time points within the same treatment. When using only the TRFLP profiles in the ordination analysis (Fig. 3a), a more evident change occurs in the bacterial community composition following harvest. This may indicate that some changes were indeed induced by the removal of the crops, but that these changes primarily concerned rare bacterial taxa, the distribution of which could not be statistically related to a particular sampling occasion. It is further possible that rarer taxa in the soil samples may have been overlooked, due to limitations in the effort of sampling and cloning. However, because many clones were found to be associated with the same sequence types, it appears that our clone libraries contain most of the dominant taxa occurring in our samples. Despite these limitations, a change over time could still be detected in the bacterial community composition, although the contribution of crop removal to these changes, compared with that induced by other time-related factors (e.g. climatic conditions or plant growth), remains unclear. Autocorrelation between several factors analysed in this study makes it is difficult to attribute changes in microbial communities to a single factor. Interestingly, dramatic changes in AM and bacterial communities were well correlated with differences in above ground production between the different fertilization regimes. Presumably, increased plant growth would influence microorganisms that rely directly or indirectly on below ground carbon allocation of plants. Additionally, it would be expected that crop harvesting interrupts the allocation of photosynthates belowground, and that this has a direct impact on root-associated microorganisms: the disappearance of AM fungi may result in a reduced frequency of occurrence of bacteria associating with the mycorrhizal fungi or bacteria depending on carbon provided by living mycorrhizal roots. An additional expected effect of crop removal is a sudden availability of carbon in the form of senescing roots and mycorrhizal fungal mycelia in the soil, which may favour other groups of microorganisms with cellulolytic or chitinolytic capabilities.

Concluding remarks

In general, application of organic fertilizers influenced both the community composition and the taxon richness of AM fungal and root-associated bacteria. Among the different treatments with either organic or mineral fertilizers, sewage sludge application resulted in the most incongruent microbial communities, showing a decline in AMF richness and a significantly altered bacterial community in terms of a higher bacterial richness and an overrepresentation of certain bacterial groups. This is consistent with the findings of Esperschutz *et al.* (2007), and may indicate that rhizosphere bacteria are more sensitive to changes induced by fertilizer amendments than are AM fungi. It should, however, be noted that we analysed AM fungi colonizing the

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roots; differential effects of fertilizers on fungal extraradical mycelia may be more noticeable compared with effects on intraradical colonization. When comparing the organic fertilizer treatments in the present study, amendment with green manure resulted in the highest richness of both AM fungi and soil bacteria, compared with control plots or plots amended with farmyard manure. Mineral fertilizers significantly influenced the community composition of AM fungi and ascomycetes in maize roots, and bacteria in root-associated soil aggregates. In particular, ammonium sulphate application resulted in significant reductions in plant growth and richness of both AM fungal- and bacterial taxa, although a small number of bacterial taxa appeared to increase under these conditions. The observed changes of AM fungal and bacterial community composition in the ammonium sulphate treatment largely appear to be linked to a decline in soil pH induced by the fertilization regime. This suggestion is supported by the fact that the highest taxon richness of bacteria was observed in the calcium nitrate treatment, which also exhibited the highest pH. Soil pH has also been found to be the most important predictor of bacterial community structure on the ecosystem level, with a higher diversity associated with neutral soils and a lower diversity in acidic soils (Fierer & Jackson, 2006). However, as the highest richness values of AM fungi and an intermediate to high number of bacterial taxa were found in plots amended with organic fertilizers, it appears that other factors, e.g. soil carbon content, phosphate and above-ground production, may contribute to an increased microbial diversity in agricultural soils. Our study confirms that fertilization regimes may not only have quantitative effects on, for example, plant production or mycorrhizal colonization, but may also considerably influence the community composition of both bacteria and fungi associated with the plant roots. Mulching and a high soil pH thereby appear to promote microbial diversity, with possible further consequences for plant–microorganism interactions and plant productivity.

The relatively high frequency of nonmycorrhizal root-inhabiting fungi in our samples indicates that this group of fungi has been overlooked to a great extent. Very little is known about the ecology of these fungi, e.g. whether they may be mutualists, commensals or antagonists. In order to gain an insight into the spatial structure and dynamics of microbial communities, all the constitutive parts need to be acknowledged. An important aspect of this will be to improve sampling procedures and other parts of the methodology in order to characterize microbial communities in environmental samples with a fine-scale resolution.

In our study, we did not detect a significant congruence between the distribution patterns of AM fungi and bacteria across the different treatments, suggesting low levels of interaction between the two assemblages. This apparent lack of interaction may be real, but it may also be related to the problems of sampling soil and roots at a sufficiently high level of spatial resolution. Previous experimental studies have indicated that certain bacterial groups appear to associate more frequently with AM fungi (Budi *et al.*, 1999; Mansfeld-Giese *et al.*, 2002; Toljander *et al.*, 2006, 2007). It may therefore be possible that AM-bacterial interactions may result in codevelopment of AM and bacterial communities, with specific AM fungi being associated with specific groups of bacteria. With the analysis methods available presently, the investigation of such relationships under field conditions will require development of new sampling procedures so that communities of different microorganisms can be characterized at a much finer spatial and temporal scale.

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Supplementary material

The following material is available for this article online:

Fig. S1. Strict consensus tree of 40 equally parsimonious trees (575 steps) showing the different sequences of Ascomycetes obtained from the clone libraries.

Table S1. Summary of the groups of clones with identical sequences and represented by a single and common terminal in the phylogenetic trees for the Glomeromycota (Fig. 1).

Table S2. Summary of the groups of clones with identical sequences and represented by a single and common terminal in the phylogenetic tree for the Ascomycota (Fig. S1).

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