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Arbuscular mycorrhizal infection changes the bacterial 16 S rDNA community composition in the rhizosphere of maize

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Abstract Mycorrhizal and non-mycorrhizal (NM) maize plants were grown for 4 or 7 weeks in an autoclaved quartz sand-soil mix. Half of the NM plants were supplied with soluble P (NM-HP) while the other half (NM-LP), like the mycorrhizal plants, received poorly soluble Fe and Al phosphate. The mycorrhizal plants were inoculated with *Glomus mosseae* or *G. intraradices*. Soil bacteria and those associated with the mycorrhizal inoculum were reintroduced by adding a filtrate of a low P soil and of the inocula. At 4 and 7 weeks, plants were harvested and root samples were taken from the root tip (0–1 cm), the subapical zone (1–2 cm) and the mature root zone at the site of lateral root emergence. DNA was extracted from the roots with adhering soil. At both harvests, the NM-HP plants had higher shoot dry weight than the plants grown on poorly soluble P. Mycorrhizal infection of both fungi ranged between 78% and 93% and had no effect on shoot growth or shoot P content. Eubacterial community compositions were examined by polymerase chain reaction-denaturing gradient gel electrophoresis of 16 S rDNA, digitisation of the band patterns and multivariate analysis. The community composition changed with time and was root zone specific. The differences in bacterial community composition in the rhizosphere between the NM plants and the mycorrhizal plants were greater at 7 than at 4 weeks. The two fungi had similar bacterial communities after 4 weeks, but these differed after 7 weeks. The observed differences are probably due to changes in substrate composition and amount in the rhizosphere.

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Introduction

Mycorrhizal plants differ in their carbon allocation pattern from non-mycorrhizal (NM) plants in that they transfer more assimilates to the roots (Koch and Johnson 1984; Wang et al. 1989; Eissenstat et al. 1993). This is thought to result from the carbon demand of the fungus, which may incorporate 10% of the carbon transported to the roots (Fitter 1991) and from the higher respiration rate of mycorrhizal roots compared with NM roots (Kucey and Paul 1982). Mycorrhizal infection often changes the number of aerobic bacteria in the rhizosphere (Bagyaraj and Menge 1978; Ames et al. 1984; Meyer and Linderman 1986; Posta et al. 1994) and affects their growth rate (Christensen and Jakobsen 1993; Marschner and Crowley 1996). Mycorrhizal roots also differ in the microbial community composition of the rhizosphere from NM roots as some groups are stimulated while others are suppressed (Secilia and Bagyaraj 1987; Kothari et al. 1991; Christensen and Jakobsen 1993; Posta et al. 1994; Andrade et al. 1997; Fillion et al. 1999). For example, the number of fluorescent pseudomonads is often decreased by mycorrhizal infection (Ames et al. 1984; Meyer and Linderman 1986; Paulitz and Linderman 1989; Posta et al. 1994; Waschkies et al. 1994; Marschner and Crowley 1996). These differences have been attributed to changes in exudate composition and/or amount (Dixon et al. 1989; Paulitz and Linderman 1989; Kothari et al. 1991; Waschkies et al. 1994). Moreover, some studies showed that mycorrhizal fungi differ in their effect on the bacterial community composition in the rhizosphere (Secilia and Bagyaraj 1987; Paulitz and Linderman 1989; Krishnaraj and Sreenivasa 1992; Andrade et al. 1997).

In the past, most studies on the effect of mycorrhizal infection on rhizosphere microorganisms have relied on

culture-dependent techniques. However, such methods are very selective as only a small proportion of soil microorganisms can be cultured (Bakken 1985). Therefore, culture-independent methods such as fatty acid extraction (Cavigelli et al. 1995; Ibekwe and Kennedy 1998) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) (Muyzer et al. 1993; Ferris et al. 1996; Muyzer and Smalla 1998) are increasingly used for microbial community analysis.

In the study reported here, 16 S rDNA profiles were used to examine the effect of mycorrhizal infection, plant age and root zone location on bacterial community composition in the rhizosphere of maize. Although species present at low population densities are not represented in the community profiles generated by PCR-DGGE (Ferris et al. 1996; Gelsomino et al. 1999), community profile data obtained are especially useful for assessing the effects of different environmental factors on bacterial community composition, which can be statistically evaluated using multivariate analyses (Yang and Crowley 2000; Marschner et al. 2001).

Materials and methods

A quartz-sand-low P soil mix (9:1) was fertilised with (mg kg^{-1}) 1260 $Ca(NO_3)_2$, 223 K_2SO_4, 515 $MgSO_4$. $7H_2O$, 15 $Fe(NH_4)$ citrate, 9 $ZnSO_4$, 8 $CuSO_4$. One quarter of the soil was fertilised with 556 mg kg^{-1} $CaHPO_4$. $2H_2O$ (high P) while the remaining soil received 50 mg kg^{-1} crystalline $AlPO_4$ and 50 mg kg^{-1} crystalline $FePO_4$. 4 H_2O (low P). The substrate was autoclaved twice to eliminate the indigenous mycorrhizal fungi. A general microbial community other than mycorrhizal fungi was reintroduced by adding a soil filtrate (blue ribbon, pore size 2–4 µm, Schleicher and Schüll) of the low P soil used in the mix. One third of the low P substrate was inoculated with *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (BEG 107) (GM) and, one third with *G. intraradices* Schenck and Smith (BEG 110) (GI) by adding a soil-root inoculum from a barley pot culture of each fungus to the substrate (10% w/w). A filtrate (blue ribbon, pore size 2–4 µm, Schleicher and Schüll) of the GM and the GI inoculum was added to the NM plants and a filtrate of the GI inoculum was added to the GM soil and vice versa. After thorough mixing, the soil was filled into 1-kg pots and planted with uniform 5-day-old maize seedlings (*Zea mays* L., line 776) with two plants per pot. For seedling production, maize seeds were imbibed overnight in aerated tap water and then transferred into moistened filter paper rolls in a greenhouse with additional lighting (12 h 500 µE m^{-2}) and a day/night temperature of 21/19°C. The planted pots were kept in the same greenhouse and were watered regularly with deionised water. After 3 and 5 weeks, 50 ml of a nutrient solution containing (mM) 1 $Ca(NO_3)_2$, 1 $(NH_4)_2SO_4$, 1 K_2SO_4, 1$MgSO_4$. $7H_2O$, 0.4 FeEDTA, 2 $MnSO_4$, 1 $ZnSO_4$, 1 $CuCl_2$, 20 H_3BO_3, 0.7 $(NH_4)_6Mo_7O_{24}$) was added to the pots to prevent nutrient deficiency other than for P.

The plants (4 pots per treatment) were harvested after 4 or 7 weeks and the shoots and roots were separated. Shoot dry matter was determined after drying at 60°C for 3 days. For determination of the mycorrhizal infection, a root sample was cleared with 10% KOH and stained with trypan blue. The percent infected root length was measured by the grid-line intersection method (Giovannetti and Mosse 1980). Plant available P was determined photometrically after extraction with 20 mM Ca lactate (Vetterlein et al. 1999).

Root pieces (approximately 1 cm in length) with adhering soil were collected immediately after the plants were removed from the pots. Four replicate samples (one from each pot) were placed into autoclaved cold microcentrifuge tubes and then stored at –70°C until further processing. Samples included root tips (0–1 cm), the subapical zone (1–2 cm) and sections from the mature root zone at the sites of lateral root emergence (5 root pieces per zone). Total DNA was isolated from the samples by a DNA extraction method modified from Fast-Prep soil DNA kit (Bio101, Vista, Calif.). Briefly, 200 mM phosphate buffer and 10% SDS were added to the samples which were then homogenised in a bead beater (Fast-Prep, Model FP120, Bio101, Vista, Calif.) at 5.5 m s^{-1} for 30 s. Proteins were removed with a protein precipitation solution (PPS, Bio101) and the DNA was bound to a silica matrix (Binding matrix, Bio101), washed twice with an ethanol-salt solution (SEWS, Bio101) and then desorbed into sterile water. The DNA samples were stored at –20°C for further analysis. The DNA content of the extracts ranged between 5 and 40 µg DNA ml^{-1}. The DNA content of the different samples was not adjusted to the same concentration for amplification because the results were supposed to reflect the actual population density of the samples. All solutions and plasticware used for DNA extraction and amplification were sterilised before use.

Bacterial 16 S rDNA genes were amplified using the universal eubacterial primer set F984-Gc (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAA GAACCTTAC) and R1378 (CGGTGTGTACAAGGCCCGGGAACG) (Heuer et al. 1997). The GC clamp (Muyzer et al. 1993) attached to primer F984 prevented complete separation of the strands in the DGGE gel. For PCR, 5 µl of the DNA extract was added to 20 µl of PCR reaction mix composed of 0.2 µl *Taq* polymerase (5000 U ml^{-1} Appligene Oncor), 2 µl dNTPs (2 mM each) (Boehringer Mannheim), 2.5 µl 10× PCR buffer (Appligene Oncor), 0.4 µl of each primer (0.5 mmol l^{-1}) and 14.5 µl ultrapure water. DNA was amplified in a Biometra Trio Thermocycler using 35 cycles of 1 min denaturation at 94°C, 1 min at 55°C for primer annealing and 2 min at 72°C for primer extension. In the first cycle, the denaturation phase was extended to 5 min at 94°C to prevent annealing of the primers to non-target DNA. The 35 cycles were followed by a final step of 10 min at 72°C and cooling at 4°C (Heuer et al. 1997). Successful amplification was verified by electrophoresis in 1.8% (w/v) agarose gels with SyBR green I nucleic acid stain (FMC Bio Products, Rockland, USA).

DGGE was performed with 8% (w/v) acrylamide gels (16×20 cm) containing a linear chemical gradient ranging from 35% to 55% (7 M urea and 40% v/v formamide). The gels were allowed to polymerise overnight. DNA samples containing 20 µl of the PCR products were electrophoresed in 1× TAE buffer at 60°C at a constant voltage of 150 V (100 mA) for 5 h (Bio-Rad Dcode systems). After electrophoresis, the gels were stained for 30 min with SyBR green I nucleic acid stain (FMC Bio Products) (10,000 fold diluted in 1× TAE) and photographed under UV light with a video-imaging system. Band detection and quantification of band intensity was performed using Image Master (Pharmacia). A standard bacterial mix consisting of a mixture of pure culture DNA extracts from *Escherichia coli*, *Pseudomonas fluorescens*, *Bacillus megaterium* and *Gordonia* sp. was added twice on each gel. Using Image Master, two bands of this bacterial mix were assigned the same Rf values in all gels. The Rf value of a given band, therefore, reflects its position with respect to these two bands of the standard bacterial mix. DNA band intensity was normalised by dividing the intensity of each band by the mean band intensity of the gel (the intensities of all bands divided by the number of bands in the gel). Each peak represents a species or an individual group of species having 16 S rDNA sequences with similar melting behaviour. The band intensity indicates the relative abundance of the group under these PCR conditions. It should be noted that bias may be introduced by preferential DNA extraction and preferential amplification of certain microorganisms or sequences.

Community similarities based on relative band intensity and position were analysed by a canonical correspondence analysis with Monte Carlo permutation tests (Canoco 4.0, Microcomputer Power, Ithaca, USA). The Monte Carlo tests were based on 199 random permutations of the data. Community similarities were graphed by using ordination plots with scaling focused on inter-

Table 1 Shoot dry weight (g pot^{-1}), shoot P concentration (%), shoot P content (mg pot^{-1}), mycorrhizal infection (% root length infected) and plant available P in the substrate (µg g soil^{-1}) in non-mycorrhizal (*NM*) plants with high P supply (*NM-HP*) or low P supply (*NM-LP*) or plants mycorrhizal with *Glomus intraradices* (*GI*) or *G. mosseae* (*GM*) with low P supply after 4 and 7 weeks. Means of 4 replicates ± standard error. Values in the same column followed by different letters are significantly different (P<0.05)

Time	Treatment	Shoot dry weight			Shoot P concentration			Shoot P content			Mycorrhizal infection			Plant available P		
4 weeks	NM-HP	2.0	±0.04	b	0.27	±0.06	a	5.4	±2.4	ab	0	±0	a	42.0	±2.1	c
	NM-LP	1.1	±0.18	a	0.16	±0.01	a	1.7	±0.4	a	0	±0	a	8.7	±0.5	b
	GI	1.1	±0.08	a	0.18	±0.01	a	2.0	±0.3	a	85	±7	bc	2.4	±0.3	a
	GM	0.9	±0.06	a	0.22	±0.02	a	2.0	±0.2	a	93	±1	c	1.5	±0.4	a
7 weeks	NM-HP	15.3	±1.5	b	0.11	±0.03	a	16.4	±1.8	d	0	±0	a	40.6	±3.1	c
	NM-LP	6.8	±1.1	a	0.14	±0.06	a	7.9	±1.3	bc	0	±0	a	9.8	±1.3	b
	GI	6.2	±0.8	a	0.10	±0.01	a	6.1	±0.6	bc	93	±1	c	2.2	±0.5	a
	GM	7.9	±0.8	a	0.11	±0.01	a	8.3	±0.3	c	78	±5	b	1.9	±0.2	a

sample differences (Jongman et al. 1995). Quantitative factors potentially affecting community structure, such as presence or absence of mycorrhizal fungi, fungal species, root zone, plant age, shoot dry weight, % infection, shoot P content, shoot P concentration and plant available P were used as environmental data.

The results for shoot dry weight, shoot P concentration and P content, mycorrhizal infection and plant available P were compared by a one-way ANOVA using SigmaStat (SPSS, Chicago, USA). Significance was tested with the Student-Newman-Keuls test (P<0.05).

Results

The NM plants remained uninfected by arbuscular mycorrhizal (AM) fungi throughout the experiment, while the mycorrhizal plants had infection rates of 78–93% (Table1). The two AM fungi colonised the roots to a similar extent after 4 weeks, while after 7 weeks the infection by GI was higher than by GM. The colonisation was at a similar level after 4 and 7 weeks. After these times, shoot dry weight and shoot P content of the NM plants supplied with high P (NM-HP) was twice as high as in the plants supplied with poorly soluble P (NM-LP) (Table 1). However, this difference was significant only after 7 weeks. Mycorrhizal and NM plants supplied with poorly soluble P had similar shoot dry weights and P contents. Between 4 and 7 weeks, shoot dry weight increased six- to ninefold. Shoot P concentration was similar in all plants and decreased from 4 to 7 weeks. Plant available P in the substrate was highest in the NM-HP plants (Table 1). In the plants supplied with poorly soluble P, the mycorrhizal plants decreased the plant available P more than the NM-LP plants.

As an example, the DGGE of the subapical root zone after 7 weeks is shown in Fig. 1. A total of 38 different bands were detected, with 10–20 bands per sample. Of these, 60% occurred in all samples, 30% in a large portion of the samples and 10% of the bands were highly specific. The variability within the band patterns was higher in the rhizosphere of the NM roots than in the mycorrhizal roots. One band was particularly strong in the rhizosphere of GM plants.

Different clones from this band indicated that it contained at least three different sequences. Three clones had a high % identity with *Pseudomonas resinovorans*, *Holophaga/Acidobacterium* (uncultured) and *Acidovorax facilis* (data not shown).

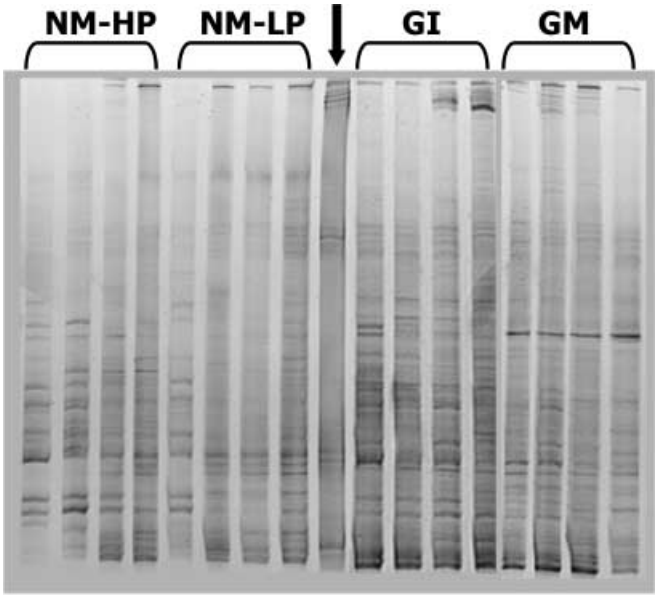

Fig. 1 Denaturing gradient gel electrophoresis (DGGE) of 16 S rDNA of bacterial rhizosphere communities in the subapical root zone of non-mycorrhizal (*NM*) plants supplied with high P (*NM-HP*) or low P (*NM-LP*) and of plants mycorrhizal with *Glomus intraradices* (*GI*) and *G. mosseae* (*GM*) after 7 weeks. Four replicates per treatment. *Arrow* indicates standard bacterial mix

Band position and band intensity were used as input for the multivariate analysis. The samples taken from the non-rhizosphere or bulk soil had a different bacterial community than the rhizosphere samples. However, because of the high root density, they could only be obtained from the top of the pots, which was drier than the rest of the soil, and were not included in the multivariate analyses presented here.

The Monte Carlo Permutation test on the DGGE patterns of the rhizosphere samples of both harvests indicated significant effects of harvest time, root zone and colonisation by mycorrhizal fungi, while P content of the shoot, shoot P concentration or plant available P in the

soil had no significant effect (Fig. 2). To investigate more subtle differences, the data were analysed for the two harvests separately (Fig. 3). Although the variation in community composition between the replicates was high after 4 weeks, the communities in the different root zones are clearly separated (Fig. 3). Even though the communities of the NM plants in the lower part of the graph and the mycorrhizal plants in the upper part appear not very well separated, the Monte Carlo Permutation test indicated a significant difference. After 4 weeks, the rhizosphere communities of the two fungi were similar. After 7 weeks, the variation between the replicates was small and clear differences between the rhizosphere community composition of the different root zones and the NM and mycorrhizal plants were found (Fig. 3). Additionally, the community composition of the GI plants differed from that of the GM plants.

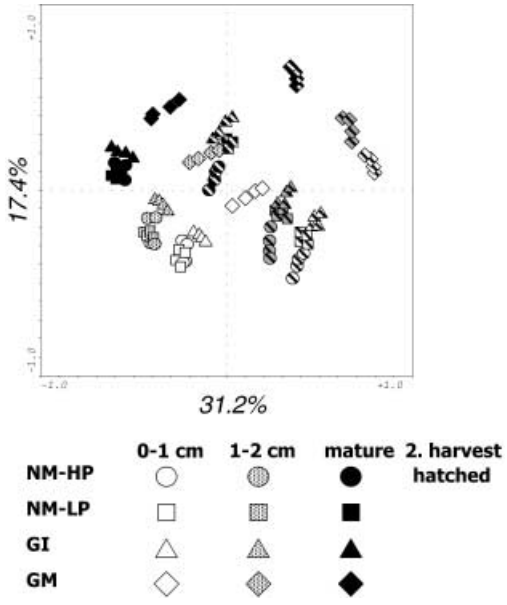

Fig. 2 Ordination plot of bacterial rhizosphere communities at the root tip, in the subapical zone and in the mature root zone at sites of lateral root emergence for NM-HP plants or NM-LP plants or for plants mycorrhizal with GI or GM with low P supply after 4 and 7 weeks generated by canonical correspondence analysis of 16 S rDNA profiles. Values on the axes indicate % of total variation explained by the axes

Discussion

This study showed that AM infection changes the bacterial community composition in the rhizosphere of maize. Additionally, the community composition is root zone specific and changes at a given root location with time. This demonstrates that the bacterial community is highly dynamic both spatially and temporarily.

The mycorrhizal effect on community composition found in this work is in accordance with earlier studies (Secilia and Bagyaraj 1987; Paulitz and Linderman 1989; Kothari et al. 1991; Posta et al. 1994; Andrade et al. 1997) and extends the effect to the large fraction of unculturable microorganisms on a microscale level. The community composition of the mycorrhizal plants differed from that of the NM-LP plants even though shoot growth and P content were similar. On the other hand, the NM-HP and the NM-LP plants had similar rhizosphere communities although they differed in shoot growth and, at the second harvest, also in shoot P concentration. Thus, the mycorrhizal effect was not the result of differences in plant growth and P status and was probably due to root colonisation by the fungus and the concomitant changes in root exudation. AM fungal infection alters root exudation qualitatively and quantitatively (Graham et al. 1981; Dixon et al. 1989; Po and Cumming 1997; Marschner et al. 1997). These changes may be due to preferential utilisation of plant exudates by the mycorrhizal fungus, exudation by the mycorrhizal fungus or changed exudation by roots due to mycorrhizal infection. It has been shown that mycorrhizal infection has profound effects on root metabolism by changing, for example, carbohydrate and protein patterns (Shachar-Hill et al. 1995; Benabdellah et al. 1998). It should also be noted that spores and sporocarps of AM fungi may contain bacteria (Filippi et al. 1998). Even though a fil-

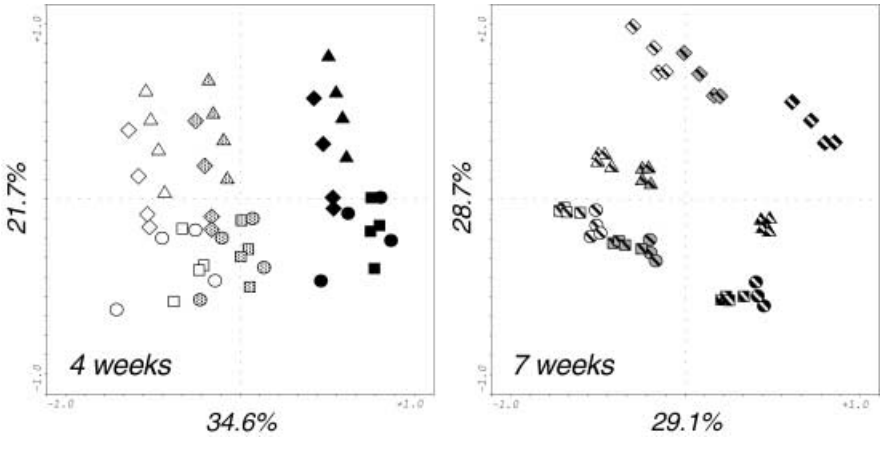

Fig. 3 Ordination plot of bacterial rhizosphere communities at the root tip, in the subapical zone and in the mature root zone at sites of lateral root emergence for NM-HP plants or NM-LP plants or for plants mycorrhizal with GI or GM with low P supply after 4 and 7 weeks generated by canonical correspondence analysis of 16 S rDNA profiles (see legend to Fig. 2)

trate of the inocula was added to the NM plants, bacteria associated with the spores may not have been transferred and could have also contributed to the observed differences between mycorrhizal and NM plants.

The difference in bacterial rhizosphere community composition between the mycorrhizal and NM plants increased with plant age. This may be due to a more specific exudate composition in older plants. But it could also indicate that the bacterial community needs time to adapt to the conditions in the rhizosphere. The latter explanation is supported by the smaller variation between the replicates after 7 weeks than after 4 weeks. It appears that a fungal species-specific effect on the rhizosphere bacterial community in maize develops with time as both fungi had similar bacterial communities after 4 weeks but these differed after 7 weeks. This was observed even though the infection rate was equally high at both harvests; however, the rate of infection of the two fungi was similar after 4 weeks but differed after 7 weeks. The differences in bacterial community composition in the rhizosphere may, therefore, be due to differences in infection rate or development of the fungus (e.g. spore formation) or could be due to species-specific effects on root exudation. A fungal species-specific effect on rhizosphere bacteria was observed in other studies (Secilia and Bagyaraj 1987; Paulitz and Linderman 1989; Krishnaraj and Sreenivasa 1992; Marschner and Crowley 1996; Andrade et al. 1997).

The temporal changes in the bacterial community composition in the rhizosphere are probably due to changes in root exudation with time. It is well-known that carbohydrate partitioning and exudation changes with plant age (Rovira 1959; Warembourg and Paul 1973). Exudation also varies along the root, with high exudation rates at the root tip and in the mature root zones at the sites of lateral root emergence (Marschner 1995). These changes may explain the observed spatial variation in the bacterial community composition in the rhizosphere. Similar root zone-specific bacterial communities were found in earlier studies using the same technique (Yang and Crowley 2000; Marschner et al. 2001). It is interesting to note that the effect of mycorrhizal infection on the rhizosphere community was observed even at the root tip, where mycorrhizal infection is usually low (Smith et al. 1992), (Figs. 2, 3). This indicates that mycorrhizal infection may alter the conditions in the rhizosphere via a systemic effect in the plant.

A few points should be noted when interpreting DGGE patterns. Only abundant species are detected in DGGE as the detection limit is about 10^6 cells g soil^{-1} (Gelsomino et al. 1999). Since the bands are separated out by their denaturation characteristics and GC content, they may contain more than one species and are, thus, an underestimation of species number. This was indicated by cloning and sequencing the predominant band in the GM samples. The three clones from this band belonged to three taxonomically different species. This is in accordance with another study using DGGE (Yang and Crowley 2000). On the other hand, some species contain several copies of the 16 S rDNA operon and can generate several 16 S rDNA PCR products, which leads to an overestimation of species number. Usually band intensity differences between species cannot be used as an indicator for species abundance (Muyzer and Smalla 1998; Gelsomino et al. 1999). However, if the intensity of a given band increases or decreases in different samples, this does indicate a relative increase or decrease in abundance of this species (unpublished results). Despite these points and the bias from preferential DNA extraction and amplification, PCR-DGGE offers new perspectives for assessing shifts in microbial community composition, since the ability to use small amounts of soil allows examination of root microsite effects. Furthermore, it is possible to analyse quickly large numbers of samples without the artefacts of culture-based techniques.

This study confirms previous observations from culture-based analyses that mycorrhizal infection affects rhizosphere community composition. The establishment of a highly specific rhizosphere community may have important impacts on plant nutrition and health. By favouring certain species and reducing the abundance of others by inoculation with selected mycorrhizal fungi, the population density of plant-beneficial microorganisms or plant pathogens may be manipulated.

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